AI-2 communication in *Staphylococcus epidermidis*:

A study on possible effects of thiophenones

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Abstract

Staphylococcus epidermidis has developed from a skin and mucus commensal to an opportunistic pathogen, and is frequently causing infections in medical implants. Its ability to form biofilm on implant surfaces makes these infections extremely persistent, and re-operation is in most cases the only option to clear these infections. Quorum-sensing, chemical cell-to-cell communication between bacteria has shown to regulate several virulence factors in important pathogens, such as biofilm formation and toxin production. Molecules with the ability to quench this quorum sensing could therefore lead to new therapies, targeting traits crucial for bacterial pathogenesis. Thiophenones are quorum sensing inhibitors, showing ability to inhibit AI-2 mediated communication. When using thiophenones a decrease in biofilm formation in *Vibrio harveyi*, *Escherichia coli* and *S.epidermidis* has been observed.

The aim of this study was to investigate the effect of different thiophenones on virulence factors in *S.epidermidis*. The effect of thiophenones on *S.epidermidis* biofilm formation in combination with antibiotics was tested, as well as persister cell formation and adherence to eukaryotic cells. An in vivo model for studying Staphylococcal infections was also established using *C.elegans*. This model was used to look at the effect of a thiophenone on recovery of *C.elegans* after infection.

All thiophenones tested showed inhibition of AI-2 communication in *V.harveyi* in a bioluminescence assay. Thiophenones in combination with antibiotics did not show to have any additive effect on *S.epidermidis* biofilm formation. To isolate and test the effect of thiophenones on persister cells proved difficult, and no conclusion regarding the effect of thiophenones could be drawn. *S.epidermidis* showed low binding to Caco-2 cells, and it was therefore difficult to determine if thiophenones had any effect on the ability to bind to eukaryotic cells. Two *S.epidermidis* strains and one *S.aureus* strain was tested for their ability to cause infection in *C.elegans*. Only worms grown on *S.aureus* showed decreased survival after 7 days of infection. The effect of a thiophenone on *C.elegans* recovery from 24 h infection by *S.aureus* was tested, but no persistent infection was detected. It was therefore difficult to make any conclusion about the effect of thiophenone on recovery, but the thiophenone concentration was shown not to be toxic for the worms.

Optimization of the different methods is needed to make any conclusions on thiophenones effect on the different virulence factors in *S.epidermidis*.

Abbreviations

Agr	Accessory gene regulator
AHL	Acyl-homoserine lactone
AIP	Autoinducer peptide
AI-2	Autoinducer-2
BA	Bioluminescence assay media
BHI	Brain heart infusion
BSIs	Nosocomial blood stream infections
C.elegans	*Caenorhabditis elegans*
CFU	Colony forming unit
CoNS	Coagulase-negative Staphylococci
DPD	4,5-dihydroxy-2,3-pentanedione
E.coli	*Escherichia coli*
HAIs	Healthcare-Associated Infections
HI	Heart infusion
MIC	Minimal inhibitory concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*

NGM	Nematode growth medium
PBP	Penicillin binding protein
PBS	Phosphate-buffered saline
PIA	Polysaccharide intercellular adhesin
QS	Quorum sensing
SAM	S-adenosylmethionine
S.aureus	*Staphylococcus aureus*
SCCmec	The staphylococcal cassette chromosome *mec*
S.epidermidis	*Staphylococcus epidermidis*
TF	Thiophenones
TSA	Tryptic soy agar
TSB	Tryptic soy broth
V.harveyi	*Vibrio harveyi*
w/o	Without
2.5 P	Optimized Buffered Peptone Water

Table of contents

1 Introduction

1.1 Staphylococcus epidermidis

Staphylococcus epidermidis (*S.epidermidis*) is a gram positive and coagulase negative bacterium. Coagulase-negative staphylococci are often collectively termed CoNS (or CNS), with *S.epidermidis* being the clinically most important of these (1-3). *S.epidermidis* has primarily been regarded as an innocent skin and mucosal commensal living on healthy human skin, but with medical progress leading to widespread use of indwelling medical devices it has developed into an opportunistic pathogen (3). *S.epidermidis* usually only cause infection in immuno-compromised patients or patients undergoing surgery, and is therefore a frequent cause of nosocomial infections (4). CoNS alone account for 11.4% of all healthcare-associated infections (HAIs) in the US and 31% of all nosocomial bloodstream infections (BSIs) (5, 6). Joint arthroplasties, left ventricular assist devices, intravascular catheters and cardiac pacemakers are just some of the medical devices offering new surfaces for the bacteria to colonize (2, 7, 8).

The reasons for the resilience of *S.epidermidis* infections are several. One of them is the ability to grow in thick agglomerations called biofilm on both biotic and abiotic surfaces, which makes the infection much more persistent (9, 10). Biofilm growth makes the bacteria up to a 1000- fold more tolerant to antimicrobials than planktonic grown cells (11, 12), and provides protection from components of the hosts immune system (13). Another issue is that a high percentage of *S.epidermidis* isolated from clinical settings carries methicillin resistance genes which also provide resistance toward other β-lactam antibiotics (14, 15). Resistance towards other classes of often used antibiotics also seems to be spreading among the clinical isolated strains (16, 17). The fact that the infections are difficult to clear with antibiotics leads to prolonged hospital stays and re-operation is in most cases the only option to clear the infection (2, 18). The prolonged hospital stays for the patient and the need for new surgery has great economic impacts. Alone periprosthetic joint infections caused by methicillin-resistant staphylococci have been calculated to have a cost of $107 264 per case in the US, and with an expected number of 3.5 million total joint arthroplasties carried out by 2030, the costs are enormous and increasing (7).

1.2 Pathogenicity and virulence factors

The pathogenicity of bacteria is their ability to cause disease in a host. To accomplish this they require some virulence factors which here will be defined as *"genes and proteins that facilitate the establishment of infection and persistence of the organism in the human body"* (10). Common virulence factors among pathogens are invasion, production of toxins, serum resistance, motility and antiphagocytic mechanisms (19).

With *S.epidermidis* not being a specialized pathogen, but a regular skin and mucosal commensal, the key virulence factors are also important for the commensal lifestyle (10).

1.2.1 Biofilms

The main virulence factor of *S. epidermidis* is the ability to form biofilm (4). A biofilm is a 3-dimensional agglomeration of bacterial cells on a surface, enclosed in a matrix composed of exopolysaccharides, proteins and extracellular DNA (eDNA) (20, 21). Bacterial biofilms are responsible for as much as 65% of infections in developed countries, and are in many cases the cause of persistent infections such as endocarditis, pneumonia in cystic fibrosis patients, infections related to orthopedic devices, contact lenses and urinary and central venous catheters (21-23). As briefly mentioned above, *S.epidermidis* can form biofilm both on abiotic surfaces such as indwelling devices as well as on biotic surfaces such as living tissue. Biofilm-growth is advantageous both for a skin commensal and for an opportunistic pathogen, because it provides protection from environmental stress, being scraped or washed away and also from host immune system components (24). The 3-dimensional appearance is not the only factor that separate the nature of planktonic and biofilm forming bacteria. There has also been found great differences in the gene expression. *S.epidermidis* in biofilm have been found to down regulate genes for transcription, translation and aerobic metabolism and up-regulating genes favoring fermentation and also expression of osmoprotective factors especially important for its commensal lifestyle on human skin (25). As more knowledge about the structure of biofilm is obtained, the more of its complexity is revealed.

S.epidermidis is one of the best studied clinically relevant biofilm-forming bacterium and there is therefore a fair amount of knowledge available about the biofilm formation process (26). The formation of biofilm can be viewed as a three-stage process starting with attachment, followed by maturation of the biofilm and finally detachment of bacterial cells (Fig.1) (9, 26). The primary step in the attachment is adhesion of the bacteria to a surface-

material and is normally a nonspecific process relying on forces between the surface material and the surface of the bacterial cell, such as hydrophobic interactions between polyethylene plastic and the bacteria (27, 28). When an implant or an indwelling medical device is inserted into the body, it quickly gets coated with host matrix proteins that serve as receptors for the bacteria (24). Adhesion to these proteins is probably the most important factor in initial attachment. *S.epidermidis* expresses a variety of adhesins called MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) that have the ability to bind to host matrix proteins like fibronectin, vitronectin, fibrinogen and collagen (29, 30). In addition, proteins mediating adherence directly to both the surface material and matrix proteins have been identified, like the autolysin AtlE witch mediates attachment to a polystyrene surface and also binds to vitronectin (31). Thus, it is likely that the ability to bind to both host matrix proteins and directly to surface polymers is an important function for the pathogenesis of *S.epidermidis*, and a significant trait making them the supreme colonizer of indwelling devices.

Biofilm maturation involves intercellular adhesion between the bacteria forming large cell clusters, and disruptive processes leading to the structuring of the biofilm (24). The most important molecule in the adhesion process in *S.epidermidis* is PIA, for polysaccharide intercellular adhesin, synthesized by the products of the IcaADBC operon (32-34). PIA synthesis has been shown to be an important factor for other clinically significant traits important for biofilm assembly. It has been shown that PIA-producing strains have higher adherence to host cells than a mutant strain unable to produce PIA and that the PIA-producing strain also had a higher tolerance to antibiotics (35). In addition, it has been demonstrated that PIA production protects *S.epidermidis* from components of the innate immune system like phagocytosis and antibacterial peptides (36). The other important step in the maturation process is structuring of the biofilm. The structuring of the biofilm is dependent on disruptive processes leading to the formation of the "mushroom"-like structure and complex structures like fluid filled channels for exchange of nutrient and metabolic waste products (37, 38).

The final step in biofilm life cycle is detachment of single cells or clusters of bacteria from the biofilm. This process is dependent on parts of the matrix detaching from the rest of the biofilm, for instance through mechanical forces or enzymatic activity breaking down matrix molecules (26). The detachment process in *S.epidermidis* seems to be regulated by the accessory gene regulator quorum sensing system *agr* (39). It has been shown that *agr* mutants produce a thicker biofilm and is less invasive than biofilm produced by wild type, because *agr*

is involved in regulating the detachment process (40). Detachment leads to dispersal of the infection through the blood-stream causing sepsis or re-colonization and the formation of biofilm on a second site in the body (24).

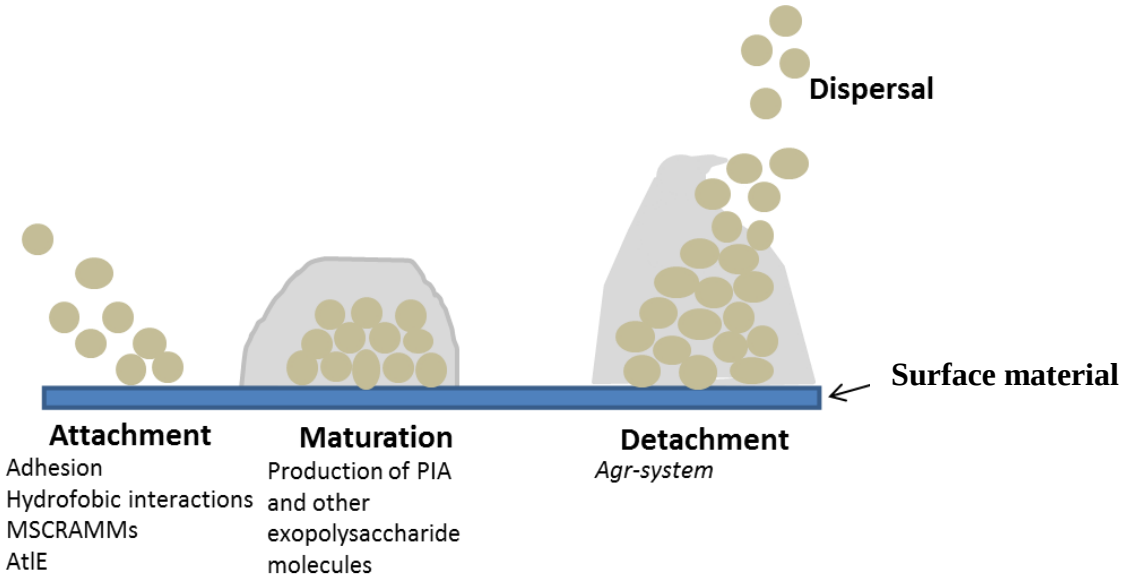

Figure 1: The formation of biofilm in *Staphylococcus epidermidis* (Figure by Gro Herredsvela Rørvik).

1.2.2 Persister cells

Persister cells are cells in a bacterial population that survive treatment with antibiotics by other mechanisms than genetic resistance (41). The existence of persister cells have been hypothesized ever since World War 2, when the army doctor Joseph Bigger found that penicillin failed to completely sterilize staphylococcal infections (42). His work has gained new attention the last decades as we have been provided evidence for the existence of persister cells and as we in general have become more aware of the limits of antibiotics (43). Persister cells are also called phenotypically tolerant cells because they are tolerant to antibiotics by mechanisms different from genetic acquired resistance. Persisters and genetic antibiotic resistant cells can be separated by the fact that genetically resistant cells are capable of growth in the presence of antibiotics, and that persisters do not have an increased MIC compared to normal cells (44). Persisters make up a small, phenotypic distinct part of an isogenic bacterial population, which survives antibiotic treatment even at very high concentrations (45, 46). The reason for their persistence seems to be that they enter a dormant, non-dividing state with slow growth and low metabolic activity (47). Since most of our

antibiotics targets dividing and metabolic active cells, they fail to target these persister cells (23, 48). The persisters can switch from the dormant state to regular growth when the antibiotic treatment stops, and thus the infection re-establishes. This new population originating from the surviving persisters is just as sensitive to antibiotics as the original population, indicating that the tolerance is not heritable (44). This capability to resume regular growth after termination of antibiotic treatment makes these infections incredibly difficult to treat, and are in many cases thought to be responsible for recurring, chronic infections (46, 49). A study in *Pseudomonas aeruginosa* strains isolated from Cystic fibrosis patients showed that the later collected isolates contained a 100-fold increase in persisters compared to earlier isolates (49). The explanation for this was found to be the formation of high persistent mutants, called *hip*, and that formation of persisters is responsible for the recalcitrance of these infections (49). For Cystic Fibrosis patients chronic infections with *Pseudomonas aeruginosa* is often mortal (50).

The occurrence of persister cells has been shown in several distinct bacterial species such as *Echerichia coli*, *Salmonella*, *P. aeruginosa,* and also populations of *S.epidermidis* has been shown to include cells with this phenotypic resistance (45, 49, 51, 52). The mechanism responsible for making a small part of a large bacterial population tolerant to antibiotics is still leading to more questions than answers. Several mechanisms have been proposed, and there is a possibility that several different mechanisms may be responsible. The possibility also exists that different mechanisms may lead to persistence against different antibiotics as indicated in E.*coli* (51). Stochastic variation, triggering of the SOS response, toxin-antitoxin expression patterns and chemical signaling are all mechanisms thought to cause a persistent population (53). Until now, only a few studies have been conducted on persister cells in *S.epidermidis*. One study has provided evidence for the existence of persister cells in S.*epidermidis*, showing that treatment with high doses of the antibiotics vancomycin and levofloxacin give a small population of 8.21×10^{-5} % and 3.09×10^{-7} % persisters, respectively (45). The same study also showed that the number of persisters were highest in stationary phase cultures and that biofilm exposed to vancomycin had an astonishing 94% persister population (45). As previously mentioned, one of the greatest challenges with bacteria in a biofilm is that they are highly tolerant to antibiotics. Another study in P.*aeruginosa* showed that the biofilm gives a protective environment for high levels of persisters, and that the bulk of the cells in the biofilm are just as easily eradicated by antibiotics as regular cells when in planktonic culture (54).

1.3 Antibiotics

The antibiotic era started in 1928 when Alexander Fleming discovered the bacteriostatic effect of penicillin (55). It was used for the first time in the 1940s for treatment of wounded soldiers during World War II, and not long after it was made available for civilians (56). The discovery of antibiotics was crucial in the treatment of bacterial infections and the mortality caused by infectious diseases declined significantly after antibiotics were clinically introduced (57).

1.3.1 Mechanism of action of selected relevant antibiotics

Antimicrobial compounds target vital cell processes in bacteria, targets that are unique for prokaryotes, thereby causing no or limited harm to the host`s own cells. Cell wall synthesis, translation, metabolism, transcription or replication are the main targets (48). These processes in bacteria involve molecules that are sufficiently different from their eukaryotic homologues or not found in eukaryotic cells, like peptidoglycan in the cell wall and enzymes catalyzing the biosynthesis of it, and are therefore perfect as antibiotic targets (58). Antibiotics are usually classified into classes or families based on their target and chemistry, and we also distinguish between bacteriostatic antibiotics which inhibit growth, and bactericidal antibiotics that kill the bacteria (59). The first antibiotics discovered were natural products like penicillin from a penicillium mold and streptomycin from the *Streptomyces* species (55, 60). Now most antibiotics discovered are synthetic or synthetic derivatives of earlier discovered antibiotics (61).

β-lactam antibiotics

Methicillin is a β-lactam antibiotic, belonging to the same class as penicillin and the oldest class of antibiotics (62). Methicillin is a semi-synthetic penicillin, and was developed in 1960 as a treatment to the emerging penicillin-resistant staphylococci (56). The β-lactams exerts their effect by inhibiting enzymes in cell wall biosynthesis, subsequently leading to bacterial cell death (62). They bind and inactivate important enzymes, for example transpeptidases, which are responsible for the final cross-linking of peptidoglycans (62). The β-lactams bind to the bacterial cell via proteins collectively called penicillin-binding proteins or PBPs (48, 63).

Fluoroquinolones

Levofloxacin is a second generation fluoroquinolone (64). Levofloxacin is a chemically synthesized antibiotic that has been approved for use since 1996, and is one of the newer antibiotics (64, 65). The fluoroquinolones exert their effect by binding to and inhibiting two enzymes involved in replication and transcription (48). These enzymes are unique to prokaryotes, the DNA gyrase and DNA topoisomerase IV (66). DNA gyrase catalyzes the formation of negative supercoiling of DNA ahead of the replication fork (64). Gyrase comprises the two A and two B subunits encoded by the genes *gyrA* and *gyrB (64)*. DNA topoisomerase`s main function is to separate the two daughter chromosomes after replication (64). DNA topoisomerase is also composed of two C and E subunits, encoded by the genes *parC* and *parE*. Levofloxacin, like the other fluoroquinolones works by binding to the DNA-enzyme complex, and thereby halting normal DNA replication and separation of daughter strands and eventually leading to cell death (66).

Glycopeptide antibiotics

Vancomycin is a glycopeptide antibiotic and like the β-lactam antibiotics it targets peptidoglycan biosynthesis and is only used to cure infections caused by gram-positive bacteria (67). Vancomycin forms a complex with the peptidoglycan precursor units and thereby prevents the peptidoglycan polymerase and transpeptidase from cross-linking the peptidoglycan (68). Vancomycin is a natural antibiotic isolated from the bacteria *Streptomyces orientalis* and became available for clinical use in 1958 (68). It quickly became an important drug, but because of impure preparations containing fermentation products it was also known to be toxic, and was soon replaced by another new drug, methicillin (68). It was not until the 1980s that vancomycin got its renaissance because of the appearance of methicillin-resistant *Staphylococcus aureus* and penicillin-resistant *Streptococcus pneumonia (69).* Vancomycin is the primary antibiotic used to cure infections caused by coagulase-negative staphylococci and is used to treat prosthetic valve endocarditis caused by *S.epidermidis* together with rifampin or an aminoglycide antibiotic (70, 71).

Aminocoumarin

The aminocoumarin class comprises antibiotics produced by different strains of *Streptomyces*, and includes novobiocin, coumermycin and clorobiocin (72). Like the flouroquinolones, the aminocoumarin antibiotics target DNA gyrase, but through another mechanism. The aminocoumarin antibiotic binds to a domain in the B-subunit having ATP-ase activity, and since the formation of negative supercoiling requires energy from ATP hydrolysis, aminocoumarin blocks the gyrases ability to induce negative supercoiling on DNA (73). Novobiocin is the only aminocoumarin antibiotic approved for use in humans, and is primarily used against infections caused by gram positive pathogens (74).

Oxazolidinones

Linezolid is a synthetic antibiotic approved for use in 2000 and it is the first oxazolidinone antibiotic (75). Linezolid works by targeting bacterial protein synthesis, but efforts to reveal the exact mechanism behind this effect has given conflicting results (76). Early aims to determine the mechanism suggested that it worked by inhibiting the initiation phase by binding to the 30s or 50s ribosomal subunit, but later studies have shown that it binds to the ribosomal peptidyl transferase center on the 50s subunit (76-78). What is more certain is that linezolid is not cross-resistant with any other known antibiotics, and that they are very effective in targeting gram-positive antibiotic-resistant pathogens like methicillin-resistant *S.aureus* and vancomycin-resistant *Enterococcus faecium* (76, 79).

1.3.2 Antibiotic resistance

Common to all classes of antibiotics is that when they are used, bacteria will at some point develop resistance towards them. At some point bacteria have developed resistance against every single antibiotic class discovered (57). Multidrug resistant pathogens are a growing health problem world-wide, and for infections by some pathogens, available treatment options are few (80). Mutations in the antibiotic target are the most common mechanism for bacterial resistance, making the antibiotic unable to bind to the target and thereby also rendering it ineffective (58). Active efflux of the drug by the use of pumps and decreased permeability over the outer membrane are also mechanisms for drug resistance, especially in gram-negative pathogens (81). Resistance in bacteria spread because of the selective pressure antibiotic exerts on them. In a bacterial population mutations do occur, and when the population is

exposed to antibiotic treatment only the bacterial cells "fortunate" enough to have a mutation in the antibiotic target will survive and replicate, as illustrated in Fig 2 (82). Use of antibiotics does in other words select for the development of resistance and the indiscriminate widespread use of it, for instance in treatment of unserious infections and in agriculture, has contributed to speeding up this development (57, 83).

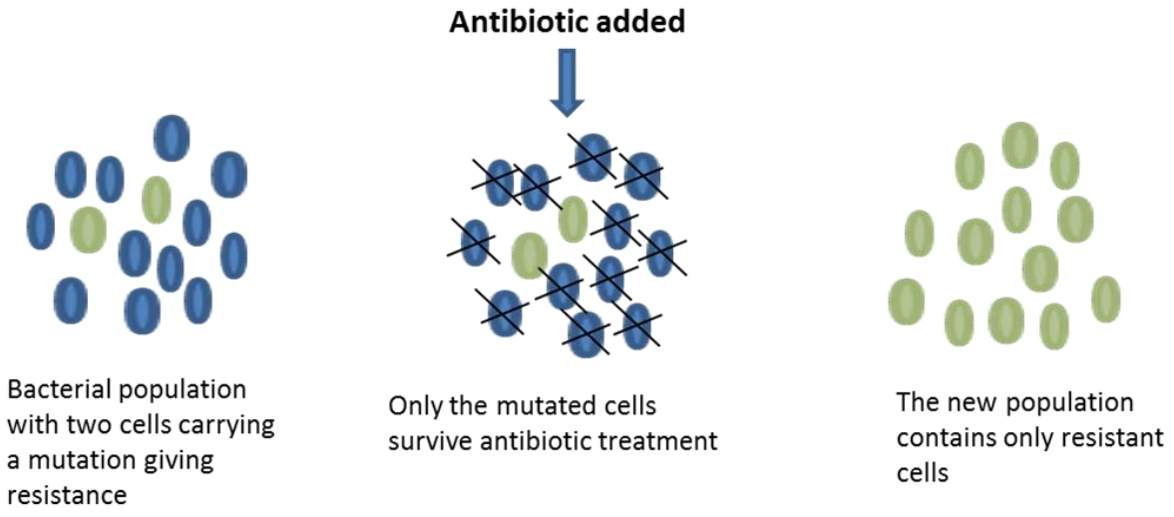

Figure 2: How resistance arises and spread in a bacterial population (Figure by Gro Herredsvela Rørvik).

Already before penicillin was widely used, clinically resistance among some bacteria was discovered, and the substance causing it was named penicillinase (63, 84). Today this penicillinase is called β-lactamases and is known to catalyse hydrolysis of the β-lactam-ring in the β-lactam antibiotics, making them ineffective (62). Methicillin-resistant *S.aureus* (MRSA) and methicillin-resistant coagulase-negative staphylococci are both β-lactam-resistant and cause major clinical problems (80). The methicillin-resistant staphylococci produce a penicillin binding protein called PBP2a encoded by the gene *mecA* carried on a genetic mobile element called the staphylococcal cassette chromosome mec (SCC*mec*) (80). This extra penicillin binding protein does not bind β-lactam antibiotics with the same affinity as the other PBPs (58). Data from around 2000 hospitals in the US in 2009-2010 showed that 43.7% of surgical site infections and 58.7% of catheter-associated urinary tract infections caused by *S.aureus* were caused by methicillin resistant isolates (5). A survey conducted from 1997-1999 also showed that MRSA isolated from hospitals in Europe was on average co-resistant to 4.5 other classes of antimicrobials (14). Another way for bacteria to gain resistance is through horizontal gene transfer of resistance genes carried on mobile genetic

elements. Coagulase-negative staphylococci like *S.epidermidis* is thought to serve as a reservoir for methicillin resistance spreading it to other more virulent staphylococci like *S.aureus* through the horizontal transfer of some subtypes of SCC*mec* *(85)*. Nosocomial MRSA increase morbidity and mortality, and with resistance to an increasing number of antimicrobials the need for means to control the spread of this pathogen is urgent (86).

Vancomycin-resistant enterococci (VRE) with *Enterococcus faecalis* and *Enterococcus faecium* being the most important species, are other multidrug resistant pathogens that are the cause of increasing problems in the nosocomial environment (80). These bacteria are known to cause endocarditis, wound and urinary tract infections and bacteremia (87). Over a quarter of the *E.faecalis* genome consists of mobile or acquired DNA, which is reflected in their ability to acquire resistance to almost all classes of antibiotics (88). Most enterococci species in addition possess intrinsic resistance to most β-lactam antibiotics and fluoroquinolones making the challenge of treating these infections even more difficult (87). Vancomycin was for a long time the only drug that could be used to eradicate enterococcus infections, but in 1988 the first cases of vancomycin-resistance was reported (89). Since then vancomycin-resistance in isolates of *E.faecium* from different health-care associated infections has been reported to be present in up to 82.6% of the isolates (5). Today the treatment options for infections with VRE are the use of combination of antibiotics from different classes and newer bacteriostatic agents like linezolid and tigecyline (80). The difficulties in treatment of VRE infections are a good example of the problems that multidrug resistant pathogens cause, and the need for new thinking in treatment of this type of infections. Bacteria quickly adapt to face new challenges, and overcoming the effects of newer antimicrobials will probably not be an exception.

1.4 Quorum sensing

1.4.1 The principles of quorum sensing

Quorum sensing (QS) is chemical cell-to-cell communication in bacteria that allows them to cooperate. They do this through production of signal molecules called autoinducers, whose extracellular concentration increases in response to an increasing bacterial population (90). According to the Oxford dictionary a quorum is the number of members of an assembly or a society that must be present at a meeting to make the proceedings of that meeting valid. This is also a good description of this phenomenon in bacteria, which allows bacteria to sense how many they are, and make social decision based on cell-population. This allows bacteria to coordinate processes that are density- dependent, like bioluminescence, biofilm formation, and virulence factors like toxin production (91, 92). These are all processes that are dependent on a certain cell density to succeed; it would have little effect if a single bacterial cell started to produce toxins in a human host or bioluminescence in a squids light organ. When the autoinducers reach a minimum threshold concentration they bind to an extracellular receptor which triggers a signal transduction cascade, eventually leading to a change in gene expression in the entire population (93). Since the quorum sensing system was first described in *Vibrio ficheri*, a bioluminescent symbiont of the squid *Euprymna scolopes* light organ in the 1970s, the view of bacteria as simple unicellular organisms has changed with an increased understanding of their cooperative behavior (94, 95).

Several different quorum sensing systems have been characterized, and they are usually classified according to the autoinducers and their respective receptors. Some of the autoinducers have high intraspecies specificity; others are used for interspecies communication (96). The system described in *Vibrio fischeri* consists of a protein called LuxI which synthesizes the freely diffusible autoinducers acyl-homoserine lactone (AHL) and a cytoplasmic autoinducer receptor called LuxR, which together control the expression of the luciferase operon required for light production (90). Homologues to this LuxIR system are also found in many other gram-negative proteobacteria and they each produce AHLs with different structure varieties and with specificity for different autoinducer receptors providing only intraspecies communication (97, 98). One of the species with LuxIR homologues with known function is *Pseudomonas aeruginosa*, having two LuxIR homologues called LasI-LasR and RhII-RhiR (99). The target genes that these AHLs regulate differ between species.

In *P.aeruginosa* they have been shown to regulate virulence determinants, production of secondary metabolites and structuring of biofilm (98-100).

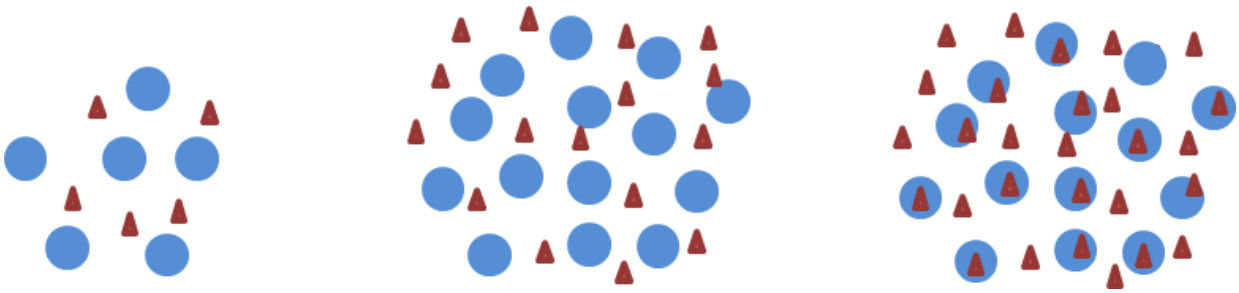

Figure 3: The basic principle of AHL-based quorum sensing - A small population of bacteria produces a small amount of AHLs which diffuse out of the cell. As the population grows, more AHLs are produced. When the concentration of AHLs in the environment reaches a certain threshold they bind to the cytoplasmic receptor in the bacterial cells, and the gene expression is changed in the population (Figure by Gro Herredsvela Rørvik).

Gram-positive bacteria on the other hand have different systems for quorum sensing, where in most cases autoinducer peptides (AIPs) are used as a signal and the receptor is part of a two-component signaling system (96). The two-component system consists of a membrane located receptor histidine kinase and an intracellular response regulator protein that regulates transcription of target genes after phosphorylation by the receptor kinase (101). In contrast to AHLs these signaling peptides need to be transported by an ATP-binding cassette exporter for the secretion to the extracellular environment (102). This system is found in several different gram positive species including *Bacillus subtilis* and *Streptococcus pneumonia*, where it regulates genetic competence and in *S.aureus* where it regulates different virulence factors (102). In staphylococcus a two component QS system encoded by the *agr* locus is found (103). The locus comprises of the genes *agrA, agrC, agrD, agrB* which are co-transcribed, as well as a gene for a regulatory RNA called RNAIII with its own promoter (103). The products of *agrB* and *agr D* combines to generate the signaling peptide which is 7-9 amino acids long and contains a thiolactone ring (104). This peptide binds to the transmembrane histidine kinase receptor encoded by *agr C*, which autophosphorylates itself before phosphorylating the product of agrA, the response regulator. agrA activates its own promoter and the promoter for

RNAIII, and thereby increases expression of the *agr* locus and RNAIII, which controls and up-regulates several genes (103). In *S.epidermidis* it has been shown that *agr* negatively regulates the production of the autolysin AtlE, involved in attachment to polystyrene surfaces, and that *agr* negative mutants had a greater capacity to adhere to the surface and thereby also increased biofilm formation (92). Even though the agr system is found in most Staphylococci differences in the AIPs and the cognate receptors leads to species specificity (103).

A more universal Quorum sensing system based on the autoinducer AI-2 is found in a number of different species. It is found in both gram-positive and gram-negative bacteria, and is thought to be used for interspecies communication (90). The autoinducer AI-2 is synthesized by the product of the *LuxS* gene, with homologues found in over 500 sequenced bacterial genomes (105). LuxS is the third enzyme in the S-adenosylmethionine (SAM) utilization pathway, in which SAM is utilized as a methyl donor in cellular processes, and is required to convert SAM to homocysteine and 4,5-dihydroxy-2,3-pentanedione (DPD) (Fig.4) (106). DPD is not thought to be the signaling molecule but an AI-2 precursor, because it is highly reactive and unstable in solution, and therefore cyclizes into a furanone ring that is thought to be AI-2 signaling molecule (106). The structure of AI-2 has only been solved for two species, *V.harveyi* and *Salmonella typhimurium* (Fig.4) (107).

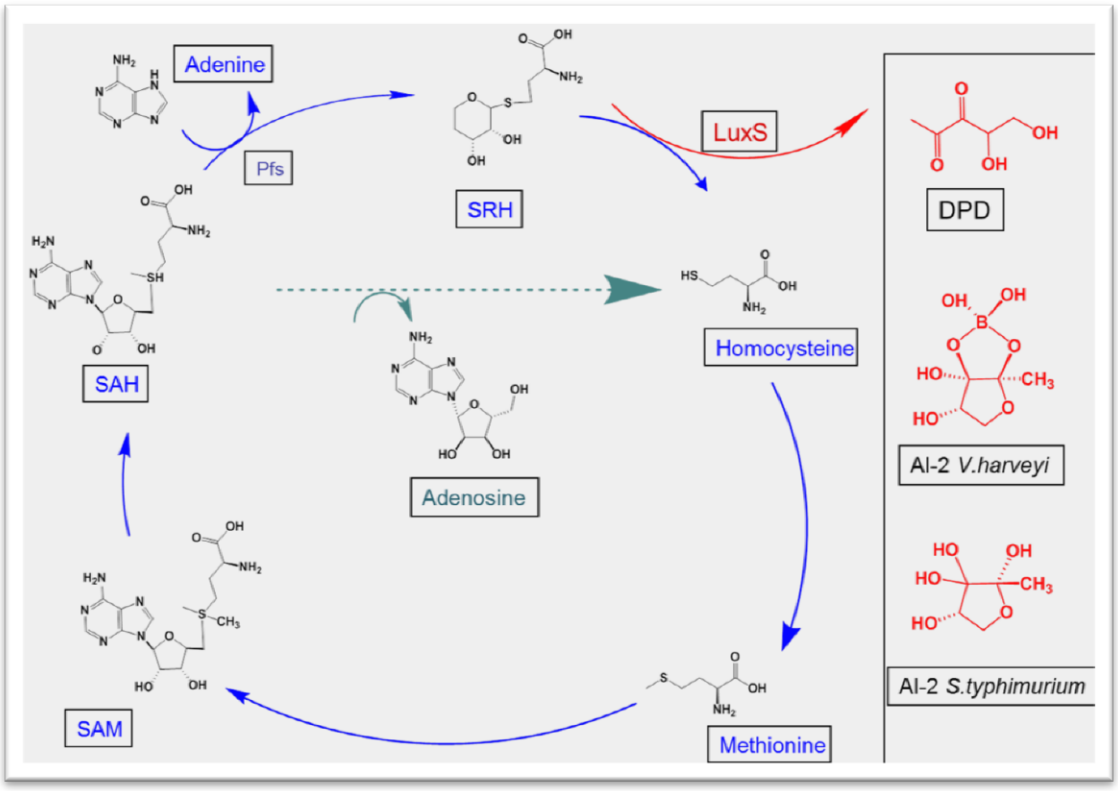

Figure 4: The activated S-adenosylmethionine cycle (SAM) and the structure of DPD and known AI-2 molecules. DPD is made as a by-product in the SAM-cycle when S-ribosyl-homocysteine (SRH) is converted to DPD and homocysteine by the LuxS synthase enzyme. The AI-2 precursor DPD can spontaneously cyclize into different AI-2 signaling molecules, as seen in *V.harveyi* or in *S.typhimurium*. Based on figure by Sun, J, et al (Figure by Anne Aamdal Scheie) (108).

The AI-2 based quorum sensing system was first discovered in *V.harveyi*. It was found that AHL-mutants unable to communicate through this AHL pathway were still capable of quorum sensing and were still producing bioluminescence in response to cell density (109). It was also found that these AHL- negative mutants responded to cell free supernatants from both marine and terrestrial bacterial species like *Yersinia enterocolitica* and *Vibrio cholerae* through AI-2 quorum sensing, leading to the conclusion that this could be an interspecies communication system (110). An interspecies communication system would give the bacteria the advantage to monitor the number of non-self-cells in the environment and coordinate behavior in response to it, in addition to the intraspecies QS systems allowing them to monitor the density of own cells (96). *S.epidermidis* is one of the species having this *luxS* homologue and is capable of AI-2 quorum sensing (Fig.5) (111). In *S.epidermidis* it has been shown that the *luxS* gene is involved in negative inhibition of PIA-production and that a luxS mutant therefore produces thicker biofilms and has an increased capacity to cause CVC-associated infections in a rat model (112) . But a study carried out with another clinical isolated strain of

S.epidermidis has shown that inhibiting AI-2 mediated communication leads to decreased biofilm formation, giving some conflicting results of the role of *luxS* in biofilm formation (113). AI-2 has also been shown to regulate genes involved in metabolism, virulence associated genes coding for lipases and the production of phenol soluble modulins (PSM) which are virulence factor that triggers cytokine release in cells of the human innate immune system potentially leading to sepsis (114, 115).

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   1 atgactaaaa tgaatgtaga aagctttaat ttagaccata ctaaggttgt tgcacctttt
  61 attcgtctag ccgggactat ggaaggtctt aatggtgatg tcatacacaa atatgacatt
 121 cgtttcaaac agcccaataa ggaacatatg gatatgcctg gtctacattc cttagagcat
 181 ttaatggcag aaaacattag aaatcatact gataaagtag tagatttaag tcctatgggt
 241 tgtcaaactg gattctatgt ttcatttatt aatcatgacg actacgatga cgtattaaat
 301 attatcgatc aaacattgca tgatgtgtta aatgctagcg aagtcccagc ttgtaatgag
 361 gttcaatgtg gttgggctgc aagtcattct ttagaaggtg ctaaaacaat tgctcaagca
 421 tttttagata aaagagagca atggaatgac atctacggag aaggtaaata a
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Figure 5: *S.epidermidis* RP62A *luxS* gene sequence (111).

1.5 Quorum sensing inhibition

Since quorum sensing regulates several virulence factors crucial for bacterial pathogenesis, like toxin production and biofilm formation, interfering with or quenching this communication could potentially lead to a new way to treat or control bacterial infections. The process of autoinducer biosynthesis, autoinducer accumulation and receptor-ligand recognition involves several molecules unique to bacteria that could serve as novel targets for a new class of antimicrobial drugs. In contrast to most of the antibiotics now in use, inhibiting quorum sensing would not directly affect fitness and thereby not conduct any selective pressure on the bacteria, and will therefore not contribute to select for drug resistant pathogens (116). Seen especially interesting, is inhibition of quorum sensing through AI-2, because this could lead to the development of broad spectrum therapies targeting both gram positive and gram negative pathogens (96). Quorum sensing inhibiting drugs could be used in combination with antibiotics, for instance in the treatment of bacteria where biofilm is a key virulence factor, hindering biofilm formation that makes the bacteria far more antibiotic

tolerant. This could possibly make the treatment of chronic infections involving pathogens with biofilm formation as a key pathogenicity property far more successful than it is today.

1.5.1 Furanones

Halogenated furanones were first isolated as secondary metabolites from the macro alga *Delisea pulchra* after it had been observed that this alga was somehow able to avoid bacterial surface colonization (117). The reason that *D.pulchra* did not have the widespread surface colonization seen on other alga was found to be that these halogenated furanones had structures resembling AHL-signaling molecules and therefore disrupted QS-signaling in bacteria (118). They were shown to be antagonistic inhibitors of AHL-mediated signaling through interference with the luxR receptor without affecting growth of the bacteria (119). These compounds was shown to inhibit several pathogenicity associated bacterial processes known to be quorum sensing-dependent, like the AHL-dependent swarming motility in *Serratia liquefaciens* and bioluminescence and toxin production in *V.harveyi (120, 121).* It was later demonstrated with the use of a *V.harveyi* QS reporter strains that one of these natural brominated furanones from *Delisea pulchra* not only inhibited quorum sensing through interference with AHL-mediated signaling, but that it also did so independently through AI-2 interference (122).

With these natural furanones as a starting point several synthetic furanone analogues with the ability to inhibit quorum sensing has been synthesized and many possible applications have been examined (123). It has been shown that biofilms grown in the presence of a synthetic furanone are more susceptible to antibiotics and that the furanone contribute to a more rapid clearance of *P.aeruginosa* infection in a mouse model (124). In *S.epidermidis* it has been shown that coating surface materials with furanones at a non-antimicrobial concentration decreases biofilm formation, showing that furanones could have a potential in avoiding implant associated biofilm infections (125, 126). Furanones has also been shown to reduce persister cell formation during growth and sensitizing the persisters to antibiotics in both *E.coli* and *P.aeruginosa* (127, 128). Together all of these results on furanones ability to control bacterial behavior associated with pathogenicity implicates that quorum quenching could have a promising future in the search for new therapies against bacterial infections.

1.5.2 Tiophenones

Thiophenones are analogues of furanones where the oxygen atom of the furanone is replaced by a sulfur atom, and like the furanones they are also able to inhibit biofilm formation (129). Several studies have compared the effect of furanones and thiophenones on different virulence traits thought to be quorum sensing dependent. A thiophenone, TF101, structurally related to a furanone has been shown to inhibit biofilm formation in *S.epidermidis* more effectively than the furanone, whereas a structurally different thiophenone, TF310, has been shown to be the most effective one (113). In the same study it was also demonstrated that both the thiophenones and the furanone were able to inhibit bioluminescence in a *V.harveyi* AI-2 reporter strain, the thiophenones being more effective than the furanone. This indicates that thiophenones are QS-inhibitors inhibiting AI-2 mediated signaling, even more effectively than furanones. A study on thiophenones ability to protect brine shrimp larvae from *V.harveyi* infection has demonstrated that the thiophenone, TF310, at concentration of only 2.5μM could completely protect the larvae from infection, without being toxic (130). A previous study showed that to achieve the same protection of the larvae from *V.harveyi*, a concentration of 65μM of a synthetic furanone was needed (130, 131). The exact mechanism of action of the quorum sensing inhibition of thiophenones is still not completely understood. Experiments where *E.coli* have been pre-treated with thiophenones have given some insight, as it was shown that this pre-treatment not affected the ability of the supernatant to induce bioluminescence in a *V.harveyi* reporter strain, indicating that thiophenones does not affect AI-2 synthesis, but rather has an effect at receptor-level (132). Many thiophenones with different substituents have been synthesized, and several of them have been shown to have biofilm inhibiting properties (133). Studies on these different thiophenones could reveal more of the potential that QS-inhibitors could have in infection control. So far, thiophenones have been shown to inhibit quorum sensing in both gram negative and gram positive bacteria and in having an effect on various virulence traits thought to be quorum sensing dependent, without being toxic.

2 Aims of the study

The main goal of this work is to study the effect of AI-2 quorum sensing inhibition using different thiophenones on various important virulence factors in the opportunistic pathogen *S.epidermidis.*

Sub goals:

- To study the ability of selected thiophenones to inhibit AI-2 based quorum sensing by using a bioluminescence assay.
- To study the effect of thiophenones alone and in combination with selected antibiotics on the formation of biofilm by *S.epidermidis*.
- To establish a method to isolate *S.epidermidis* persister cells and test whether thiophenones could revert the cells to antibiotic tolerance.
- To study the ability of thiophenone to affect *S.epidermidis* adherence to eukaryotic cells.
- To establish an infection model in *C.elegans*.

3 Materials and methods

3.1 Materials

Bacterial strains

All bacterial cultures were stored in appropriate culture media supplemented with 30% glycerol in a -80°C freezer. When needed, one of the bacteria stocks was thawed and inoculated in appropriate culture media. The rest of the stock was discarded to avoid repeated thawing and freezing.

Table 1: Bacterial strains used in this study

Strains designations/ATCC number	Genus
RP62A/ ATCC 35984	*S.epidermidis*
AMC 263/ ATCC 155	*S.epidermidis*
HFH-30172 / ATCC 1694	*S.aureus*
MM32/ BAA-1121	*V.harveyi*
OP50	*E.coli*

Thiophenones

Thiophenones were stored in absolute ethanol to a concentration of 100mM in a -20°C freezer. This stock was further diluted in tryptic soy broth (TSB) (BD Bacto™) to a working solution, and stored in a -20°C freezer. When needed the thiophenone working stock was thawed, and any remaining solution was discarded to avoid thawing and freezing.

Antibiotics

All antibiotic stocks were prepared from powder and a suitable solvent. Levofloxacin (SIGMA Life science) was diluted in 70% ethanol and novobiocin (SIGMA Life science),

linezolid (SIGMA Life science) and vancomycin (Aldrich chemistry) was diluted in d^2H_20 and stored in a -20°C freezer. It was thawed and further diluted to a working concentration in TSB when needed. Any remaining solution was discarded.

Statistics

All statistical analyses were performed using Sigmaplot 12.5.

3.2 MIC determination

S.epidermidis RP62A was grown by inoculating 10µl of frozen stock in 10ml TSB (tryptic soy broth) and grown to OD_{600nm} 0.1 at 37°C with shaking at 180rpm, approximately 4.5 h. *S.epidermidis* together with different antibiotic concentrations was added in a 96-well microtiter plate (Nunc). The antibiotic concentrations chosen were both below and above MIC values found in literature. Dilutions with 0,625 times difference between each well was achieved by adding the selected antibiotic in TSB to a total volume of 200µl in well number 1 and 75µl TSB in the other wells, and then adding 125µl from well 1 to well number 2, mixing, and then moving 125µl from well number 2 to well number 3 and so on. 75µl *S.epidermidis* culture was then added to all wells except sterile control with only TSB to a final volume of 150µl in each well. Since each well with antibiotic was diluted one time when the culture was added, the antibiotic concentration in the wells was twice of wanted final concentration. The plates were incubated at 37°C without shaking overnight (16-18 h). $OD_{600\ nm}$ was measured in a plate reader (Synergy HT, BIO-TEK®).

3.3 Thiophenones as AI-2 signaling inhibitors

Vibrio harveyi MM32 was grown in HI media (BD Bacto™) supplemented with 20g NaCl\L at 30°C with shaking 180rpm overnight, then 10% of the culture was transferred to BA-media (recipe in supplementary) and grown for second overnight. *S.epidermidis* RP62A supernatant was prepared by inoculating 5µl frozen culture in 5ml TSB, HI or BHI (BD Difco™), and incubating it for 4 h, 6 h or 16 h, at 37°C with 180rpm shaking. This was followed by centrifugation at 8000g in 10 min, and filter sterilization of the supernatant with a 0,2µm celluloseacetate filter (Whatman®) to a cell free supernatant. The assay was done by incubating *V.harveyi* MM32 together with 2.5µM of the AI-2 precursor DPD or *S.epidermidis*

supernatant in a 96-well plate (Nunclon™ DELTA Surface, Nunc™) to a total volume of 100µl, 4 replicas of each. The plate was incubated at 30°C with 350rpm shaking for 3 h (Eppendorf Thermomixer comfort), and luminescence was measured using a plate reader (Synergy HT, BIO-TEK®). Next, AI-2 signaling inhibitors TF101, TF301, TF310 or TF403 were added to a final concentration of 2.5µM, concentration was chosen by comparing the effect of 2.5µM and 5µM in an independent experiment. The bioluminescence was measured directly after application of thiophenones, the plate was then incubated at 30°C with shaking 350rpm, and the luminescence was measured every 30 min for one and a half hour. The protocol was repeated in at least two independent experiments.

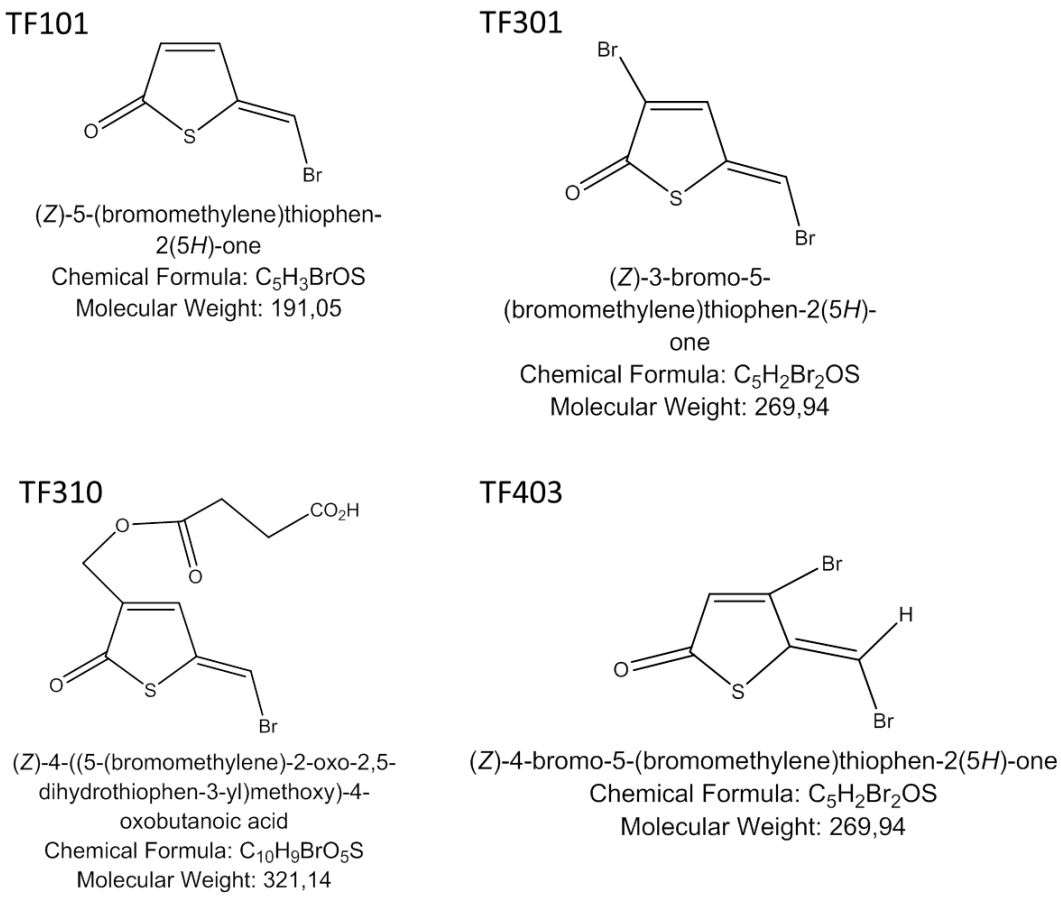

Figure 6: Structure of the thiophenones used in the experiments, TF101, TF301, TF310 and TF403.

3.4 The effect of thiophenones and antibiotic on biofilm formation

To test the effect of thiophenone and thiophenone in combination with antibiotics on biofilm formation, aliquots of 5µl *S.epidermidis* RP62A was inoculated in 5ml TSB overnight, and diluted to OD_{600nm}=0.1. Dilutions of thiophenones to a final concentration of 10µM and selected concentrations of antibiotic were made in 15ml falcon tubes, and culture was added to a final OD_{600nm}= 0,05. Samples of 400µl were transferred to 48-well plates (Multiwell™ BD Falcon™), 2-4 replicas of each sample. Samples of TSB and *S.epidermidis* culture were used as sterile/blank and growth control, respectively. The plates were incubated at 37˚C with no shaking and biofilm was allowed to form for 6 h. This was found to be the optimal time for biofilm formation in a preliminary experiment; a lot of biofilm was forming without the biofilm starting to detach (results not shown). After 6 h the plates were read at OD_{600nm} in a plate reader (Synergy HT, BIO-TEK®), to see if the thiophenone and the antibiotic affected growth of the bacteria. To quantify the biofilm, safranine staining was used (134). The supernatant was discarded, and the biofilm was washed twice in 0.9% NaCl to remove any unbound cells. The biofilm was stained with 200µl 0.1% safranine for 30 min, and then the biofilm was washed in 0.9% NaCl until all excess color was removed. The plates were allowed to dry, and 200µl 30% acetic acid was added to release bound dye. The amount of biofilm was measured by reading the plates at OD_{530nm} in a plate reader (Synergy HT, BIOTEK®).

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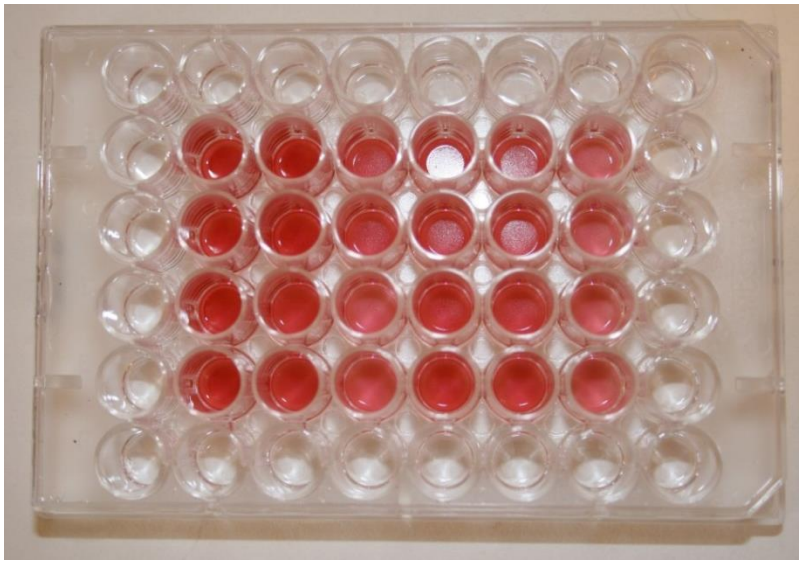

Figure 7: Safranine stained biofilm (Gro Herredsvela Rørvik).

3.5 Persister isolation

To decide the best time to isolate persister cells, and the optimal antibiotic concentration for it, *S.epidermidis* RP62A persister cells was isolated from planktonic culture as described by Shapiro *et al*, with slight modifications (45). Briefly, an overnight culture was diluted 1:1000 in TSB to an OD_{600nm} of approximately 0.01 and incubated at 37°C with shaking 180rpm. Every other hour two samples were removed from the culture. One sample got 25µg/ml or 50µg/ml of levofloxacin added, and was incubated for another 24 h, until all non-persister cells presumably were killed. The other sample was diluted in a 96-well plate and 6 replicas of 10µl were plated on tryptic soy agar (TSA), by using the drop plate method for colony counting described by Chen *et al.* to determine the original CFU/ml in the sample before antibiotic treatment (Fig.8) (135). After 24 h the antibiotic treated samples were washed twice by centrifugation at 14000g for 2 min, removing the supernatant and re-suspending the pellet in TSB. The sample was then diluted and plated out in the same way as described above. After colony counting one colony from two different time points were selected for MIC-testing, to assure that the isolated cells were persisters and not had developed resistance. The MIC testing was done as described above.

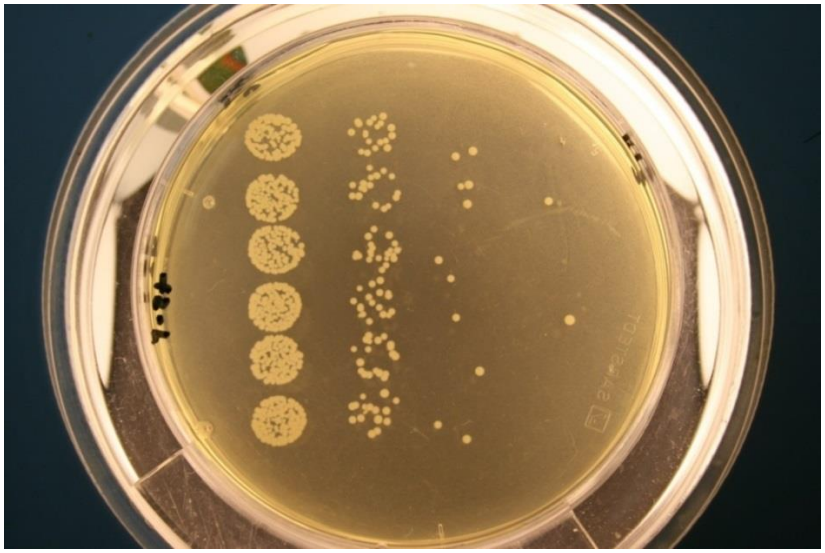

Figure 8: Drop plate method for colony counting (Gro Herredsvela Rørvik).

Optimal time for isolation of persisters was decided to be after 2 h and 6 h of growth, to compare the amount of persisters in lag phase culture with exponential phase culture, and these time points for isolation was used in further experiments. Persisters were isolated in three independent experiments with 50µg/ml levofloxacin as described above, but with samples only taken from the culture after 2 h and 6 h of growth (Fig.9).

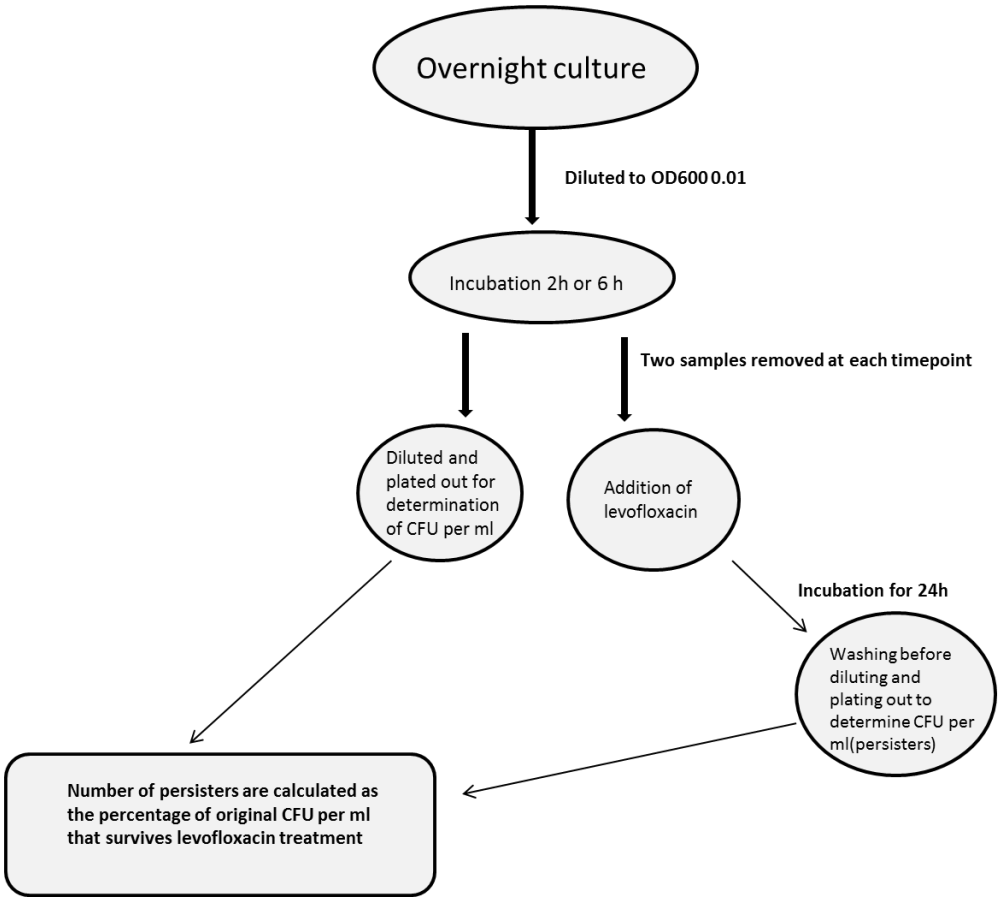

Figure 9: Flow chart showing the protocol used for persister isolation (Figure by Gro Herredsvela Rørvik).

3.5.1 The effect of thiophenones on persister formation

Persisters were isolated after 2 h and 6 h as described above. The effect of 5µM thiophenone was tested in two ways to try to establish the best method for testing their effect on persister formation. It was tested as a pre-treatment added to the culture after dilution to $OD_{600nm} = 0.05$ before incubation in 2 h or 6 h, and were not removed by washing before levofloxacin was added, and in combination with antibiotic added at the same time as levofloxacin. Persisters isolated without any form of thiophenone treatment were used as a control and untreated/pre-treated culture was used to calculate original CFU/ml. All experiments were repeated twice.

3.6 The effect of thiophenones on adhesion to eukaryotic cells

CaCo-2 cells were grown to a confluent layer in a 12-well plate, approximately 300 000-500 000 cells/well. The number of bacteria in a *S.epidermidis* RP62A overnight culture was decided by diluting it to OD_{600nm}=0.5, and counting the number of bacteria in a counting chamber (PETROFF-HAUSSER), and calculating CFU/ml. *S.epidermidis* was diluted in cell medium so that there would be approximately 40 bacteria per cell in each well. TF101, TF310 or TF403 were added to the wells in duplicate to a final concentration of 5µM, and *S.epidermidis* without thiophenones was used as a control. The plates were centrifuged in 5 min 600rpm so that the bacteria would go down to the cell layer. The plates were incubated at 37°C in 90 min. The wells were then washed three times in phosphate-buffered saline (PBS) to remove any non-adherent bacteria. Next, 1ml dH_2O was added for lysing of the cells and plates were incubated at 37°C in 15 min. The cells were then scraped with the help of a pipette tip, followed by incubation for another 15 min, at 37°C. The lysates were then collected in eppendorf tubes, and diluted from $10^{-1}$to 10^{-4} in PBS. Duplicates of 25µl from each sample were then plated out on tryptic soy agar, and were incubated at 37°C overnight before colony counting and calculations of the number of adherent bacteria to each cell.

3.7 Establishment of an infection model in *Caenorhabditis elegans*

Caenorhabditis elegans were grown on plates with nematode growth agar (NGM) (recipe in supplementary) containing an *E.coli*:OP50 lawn as food source at 15°C for 5-6 days to get adults that carried eggs. The worms were washed off the plates with M9-buffer (recipe in supplementary) and the worms were collected by adding the buffer to a 15ml conical tube and centrifuged for 1 min at 1000g. The supernatant was removed and the worms were lysed with freshly made bleach solution prepared immediately before use (3ml hypochlorite solution O.1M, 3.75ml NaOH 1M and 8.25ml d^2H_2O). The tubes were gently shaken by hand during the lysis of the worms, approximately 3 min. It was important that the lysing process did not exceed 5 min, because this could lead to destruction of the eggs. The tubes were then centrifuged at 1000g for 1 min and the pellet was re-suspended in 5ml M9 buffer. This washing step was repeated three times before the supernatant were re-suspended in 5ml M9-buffer and the eggs were incubated in room temperature overnight with gentle shaking on a

2D rotator for the eggs to hatch. The day after the L1 worms hatched from the eggs, and was harvested. They were allowed to swim down to the bottom of the tube, and most of the M9-buffer was removed. The small larvae were transferred to petri plates with NGM-agar with a lawn of *E.coli*:OP50. They were incubated for 48 h at 25°C to get L4 larvae's that were going to be used in the infection experiment.

To study infection of *C.elegans* by different bacterial strains, *S.epidermidis* RP62A, *S.epidermidis* ATCC 155 and *S.aureus* ATCC 1694 were grown by transferring a single colony to 3ml brain heart infusion(BHI) and incubating for 2 h at 37°C with 180rpm shaking, and dilute it to a OD_{600nm}= 0.1. To prepare infection plates 5µl of each strain were smeared on 6-well plates containing BHI20-NGM-agar (recipe in supplementary), 6 replicas of each. A control sample, a 6-well plate with *E.coli:*OP50 was prepared in the same way. This was used as a control because it is known that this strain is not pathogenic to the worms. The *E.coli*:OP50 plates were incubated overnight in room temperature and the others were incubated at 37°C overnight.

The L4 or young adult hermaphrodite worms that had been incubated for 48 h, were washed off the plates with M9-buffer, and collected in 15ml falcon tubes. They were washed three times by centrifuging for 1 min at 600g, removing the supernatant and then adding new M9-buffer. The washing step was repeated three times to remove all *E.coli:*OP50. Worms per 10µl were counted in a dissecting microscope (Leica MZFLIII), to determine the appropriate volume to add to each well. Between 15 and 25 worms were transferred to the lawn of the different bacteria to be tested and to control sample. Worms were allowed to spread on the wells for approximately two hours, and then the worms were counted and dead worms registered. The plates with the worms were incubated at 25°C and worms were counted in a microscope with 24 h interval for seven days in the microscope, to determine how many dead\alive worms there were in each sample. Dead worms were registered as worms which had lost their sinusoidal form, were no longer moving, and in some cases had become transparent with only the outline of the worm showing or they had a rupture in the vulva area (Fig.10). The results of the control plates were compared with the ones with the two *S.epidermidis* strains and the one with *S.aureus*, to determine if any of the strains were pathogenic to *C.elegans*.

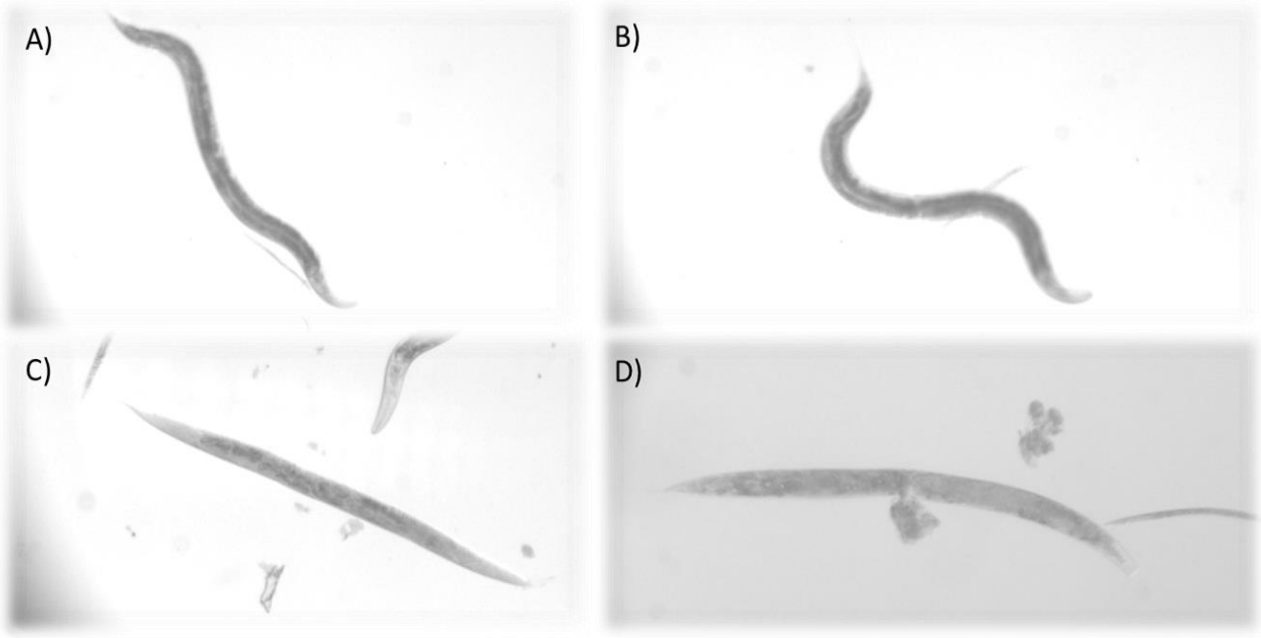

Figure 10: Separating live and dead *C.elegans*. Live, sinusoidal shaped *C.elegans* A) and B), dead, flattened *C.elegans* in C) and dead C.elegans with rupture in the vulva area D) (Micrograph - Leica MZFLIII).

3.7.1 The effect of thiophenones on *C.elegans* recovery after infection

Worms and infection plates were prepared in the same way as described above. Three control plates with 6 parallels of *E.coli*:OP50 were prepared and four plates with 6 parallels of *S.aureus* were prepared and used for infection. Approximately 50 L4 worms were transferred to each of the wells. After 24 h of infection, all worms were washed of the wells with 2ml M9-buffer, and collected in two falcon tubes. The worms were washed three times by centrifugation at 600g for 1 min, and re-suspending in 10ml M9-buffer. After washing most of the M9-buffer was removed and worms per 10µl were counted.

Recovery of the worms was tested in liquid media in a 48-well plate. 2.5P-media (recipe in supplementary) was used to test the recovery of the worms infected by *S.aureus*, and BHI-M9-media was used to test recovery/survival of the worms grown on *E.coli*:OP50. The effect of thiophenones on recovery was tested by adding 5µM TF310 to 2.5P media and as a toxicity control *E.coli*:OP50 grown worms were added to BHI-M9 media containing 5µM TF310. d^2H_2O was applied to the edges of the plate to avoid plate drying. One row remained empty due to a low number of worms, because of loss of worms between each step. Approximately 10-20 worms from the two different samples were transferred to the wells.

Dead worms were registered in a dissecting microscope (Leica MZFLIII), and the plate was incubated in a 2D rotator in room temperature. Live\dead worms were registered every 24 h.

Table 2: Plate layout for C.elegans recovery experiment.

d^2H_2O	d^2H_2O	d^2H_2O	d^2H_2O	d^2H_2O	d^2H_2O	d^2H_2O	d^2H_2O
d^2H_2O	BHI-M9+ OP50 fed worms		2.5P + OP50 fed worms	2.5P + *S.aureus* fed worms	BHI-M9+ TF310 OP50 fed worms	2.5P+TF310+ *S.aureus* fed worms	d^2H_2O
d^2H_2O	BHI-M9+ OP50 fed worms		2.5P + OP50 fed worms	2.5P + *S.aureus* fed worms	BHI-M9+ TF310 OP50 fed worms	2.5P+TF310+ *S.aureus* fed worms	d^2H_2O
d^2H_2O	BHI-M9+ OP50 fed worms		2.5P + OP50 fed worms	2.5P + *S.aureus* fed worms	BHI-M9+TF310 OP50 fed worms	2.5P+TF310+ *S.aureus* fed worms	d^2H_2O
d^2H_2O	BHI-M9+ OP50 fed worms		2.5P + OP50 fed worms	2.5P + *S.aureus* fed worms	BHI-M9+ TF310 OP50 fed worms	2.5P+TF310+ *S.aureus* fed worms	d^2H_2O
d^2H_2O	d^2H_2O	d^2H_2O	d^2H_2O	d^2H_2O	d^2H_2O	d^2H_2O	d^2H_2O

4 Results

4.1 MIC determination

The minimal inhibitory concentration (MIC) is the lowest concentration of an antimicrobial compound that will completely inhibit growth of a microorganism (136). MIC values for levofloxacin, novobiocin, linezolid and vancomycin were assayed for *S.epidermidis* RP62A. When deciding which concentrations to test, values found in the literature was used as a starting point and concentrations both below and above were tested. This was done because MIC values can vary between different test conditions, like growth media and size of inoculum, and MIC-values found in the literature could not be expected to be the same as under these experimental conditions.

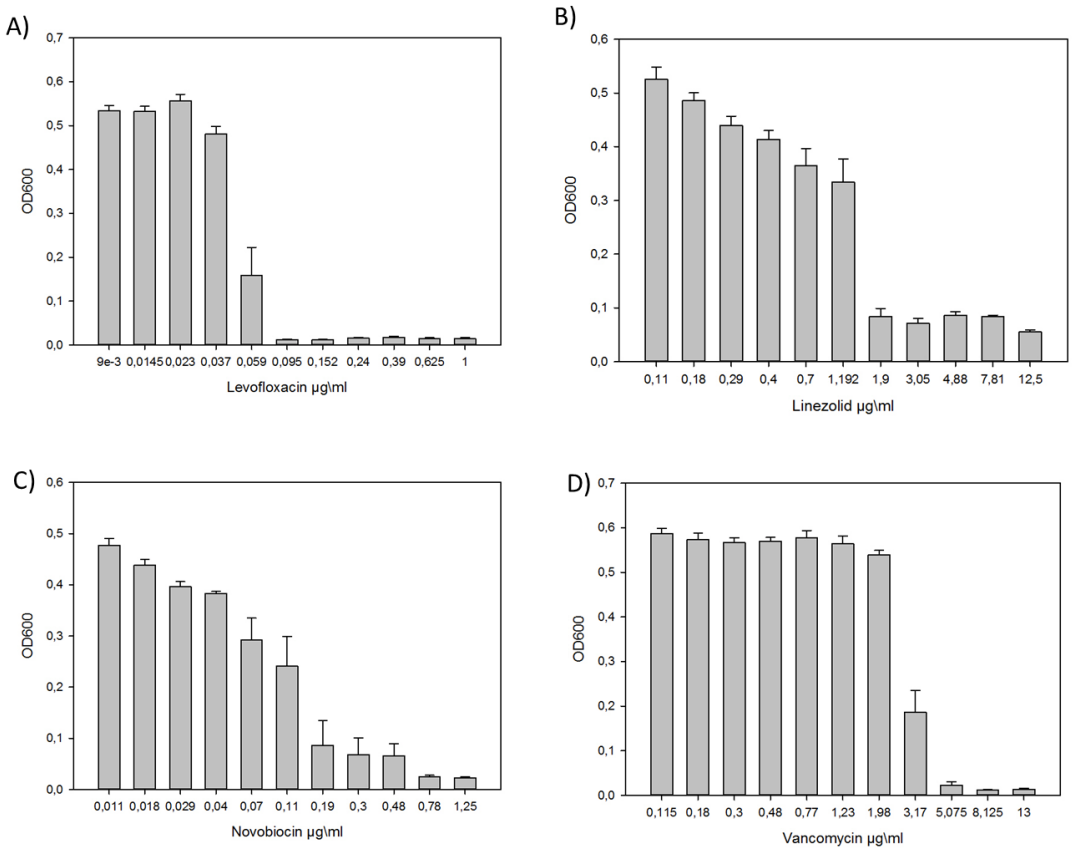

Figure 11: MIC-testing for levofloxacin A), linezolid B), novobiocin C) and vancomycin D).Result represented as mean of at least two independent experiments.

Results in Fig.11 show the mean results for all replicas from two or four experiments, with error bars representing standard error. For vancomycin, linezolid and novobiocin the experiments were repeated twice with three parallels each time. For levofloxacin the experiment was repeated four times with three parallels each time, because there were no clear results from the first two experiments. Under two of the experiments the concentration that inhibited growth was 0.095µg/ml and under the two other experiments it was found to be 0.059µg/ml. The MIC was decided to be in between these two values, around the mean which is 0.077µg/ml. For novobiocin, vancomycin and linezolid the MIC values were 0.19µg/ml, 5.075µg/ml and 1.9µg/ml, respectively. In Fig.11B) and C) it may seem like the MIC-values were not completely inhibiting growth, but this is probably due to the fact that the optical density of the inoculum before incubation were not measured and subtracted from the results.

4.2 Thiophenones as signaling inhibitors

V.harveyi MM32, a non-bioluminescence producing AI-2 reporter strain having the AI-2 receptor, was used to study the effect of thiophenones as communication inhibitor. Bioluminescence was induced in *V.harveyi* MM32 by 2.5µM DPD, and repressed by addition of 2.5µM or 5µM of the different thiophenones. All thiophenones tested inhibited bioluminescence compared to the control, at both concentrations. The results (Fig.12) shows that TF101 was the most effective, inhibiting bioluminescence at both 2.5µM and 5µM, with 98.4% and 99.5% respectively. Even the least effective thiophenone, TF310 at 2.5µM, inhibited bioluminescence as much as 83% compared to control. The results from this experiment showed that the most effective thiophenones at a concentration as low as 2.5µM was sufficient to almost completely inhibit AI-2 signaling. All samples were significantly different from control ($p<0.05$, paired t-test). Results are mean of three replicas in one experiment.

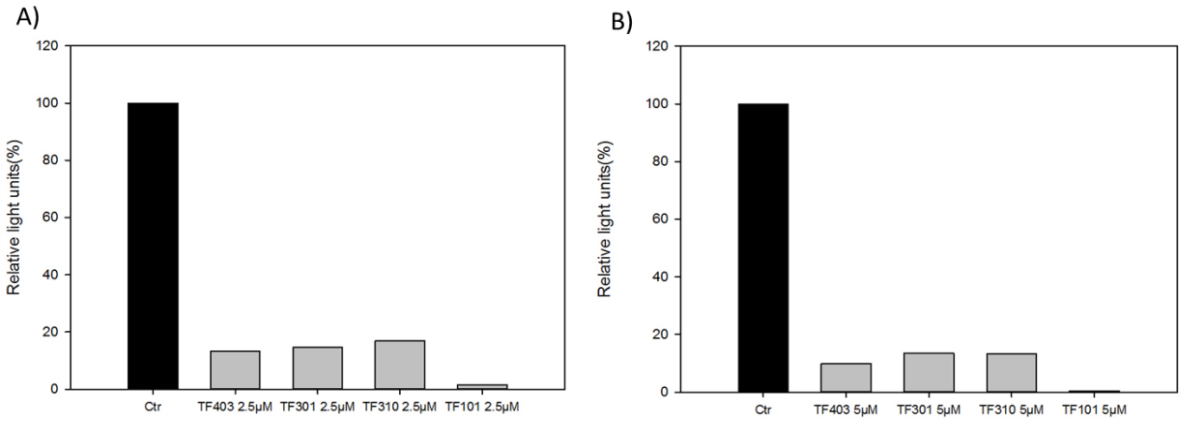

Figure 12: Effect of different thiophenones on bioluminescence in *V.harveyi* MM32at 2.5µM A) and 5µM B).

It was also attempted to induce bioluminescence in *V.harveyi* MM32 with sterile *S.epidermidis* RP62A supernatant. Fig.13 shows the results after trying to induce bioluminescence with a supernatant prepared after 4 h growth in both HI and BHI. Results are mean of four parallels with four replicas of each. There were no bioluminescence detected, compared to control where bioluminescence was induced by DPD.

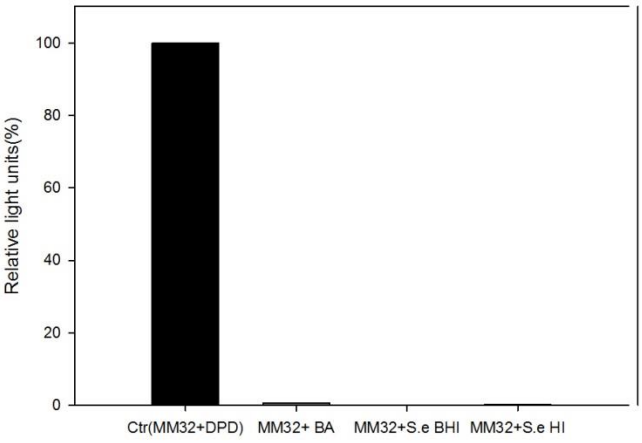

Figure 13: Bioluminescence in *V.harveyi* MM32 after induction by DPD and *S.epidermidis* supernatant prepared both from BHI and HI. *V.harveyi* MM32 with BA-media were used as negative control.

4.3 Thiophenones and antibiotic as inhibitors of biofilm formation

The effect of four different thiophenones at 10µM in combination with three different levofloxacin concentrations on biofilm formation in *S.epidermidis* RP62A is shown in Fig.14. The results show the mean of two individual experiments with 2-4 replicas of each sample, with error bars representing standard error. TF310 was the only thiophenone that made the antibiotic more effective in inhibiting biofilm formation, at all three tested concentrations. However, none of these results were statistically significant (one way ANOVA) (Fig.14C). TF403 increased biofilm formation compared to control (p=0,009, one way ANOVA). TF403 in combination with 0.15µg/ml levofloxacin inhibited biofilm slightly more effectively than the same concentration of levofloxacin alone, but this result was not statistically significant (one way ANOVA) (Fig.14D). TF101 in combination with 0,08µg/ml antibiotic (Fig.14A), seemed to inhibit biofilm formation more effectively than with antibiotic alone, but this difference was also not statistically significant (one way ANOVA).

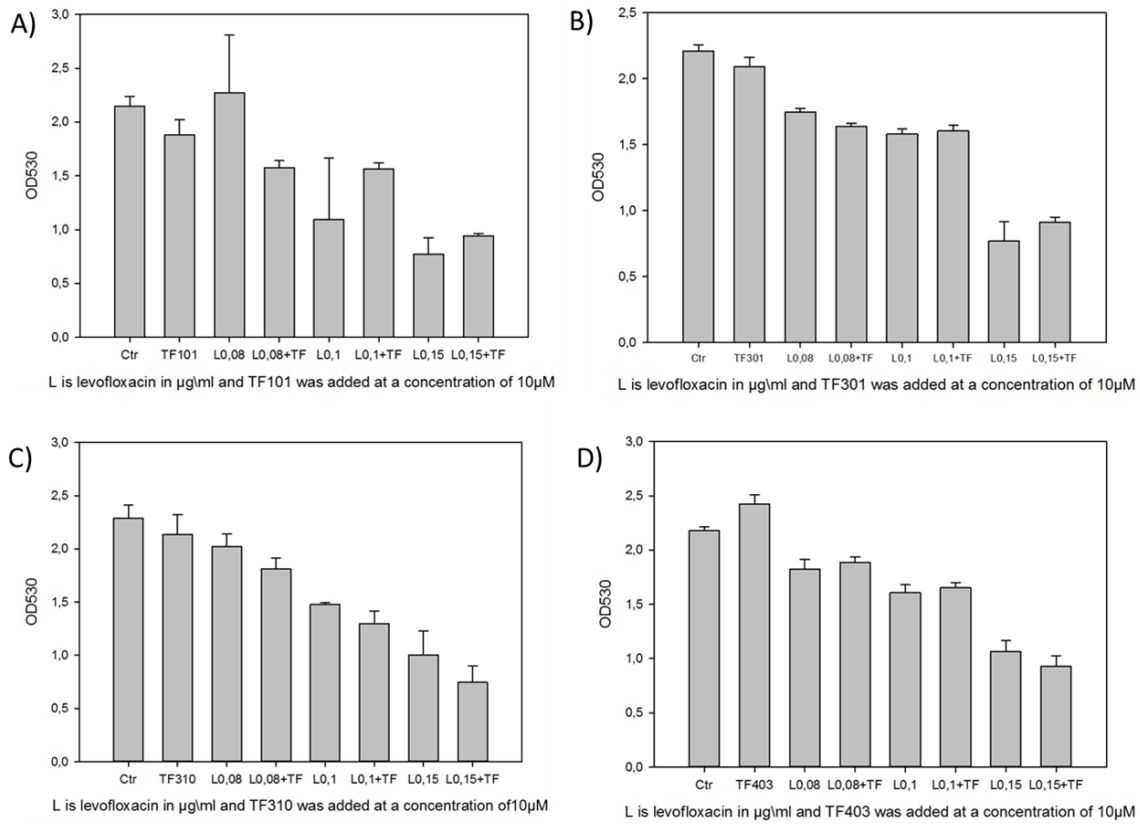

Figure 14: Effect of thiophenones TF101 A),TF301 B),TF310 C) or TF403 D) in combination with levofloxacin in inhibiting biofilm formation. Results are represented as mean of two independet experiments.

4.4 Persister isolation

To find optimal time for persister isolation and to make sure that antibiotic concentrations chosen were high enough to eradicate all non-persister cells, experiments were carried out where persisters were isolated every other hour of growth during 10 h. They were supplied with either 25µg/ml or 50 µg/ml levofloxacin (almost 325x and 650x MIC). The experiments were carried out once for each treatment, and with only one sample. Numbers of persisters were calculated as the percentage of original culture that survived the antibiotic treatment. The results from the first 4 h of growth are compared in Fig.15. The results show that from 0 h-2 h of growth, the number of persisters isolated was higher when the sample was treated with 50µg\ml than when it was treated with 25µg/ml. After 4 h the number of persisters in the two samples was more similar, $3.76x10^{-6}$ % for 50µg/ml and $4.67x10^{-6}$ % for 25µg/ml.

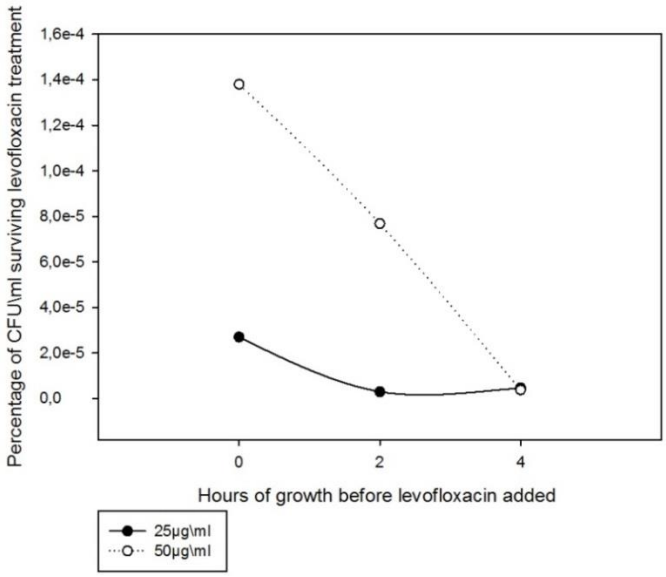

Figure 15: Comparing the percentage of persisters isolated at different time points during growth with either 25µg\ml levofloxacin or 50µg\ml levofloxacin.

MIC testing was done on two of the plates containing persisters, to assure that survival was not due to development of resistance against levofloxacin. Results are shown in Fig.16. Results of MIC-testing of persister isolated after 8 h of growth showed that the MIC was in between 0,095µg/ml and 0,152µg/ml (A). MIC-testing carried out on persisters isolated after 10 h of growth obtained a MIC-value close to 0,152µg\ml (B). The MIC-value for the isolated persister was higher than for the untreated culture, where MIC was found to be around 0.077µg\ml, but not high enough to be caused by resistance.

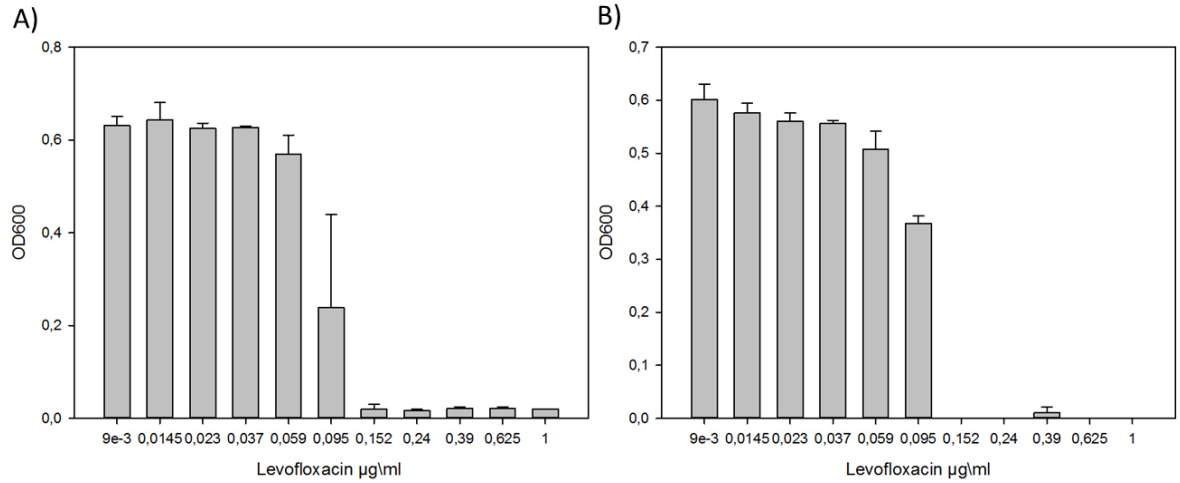

Figure 16: Shows the result on MIC-testing carried out on persisters isolated after 8 h(A) and after 10 h of growth(B).

Based on the results of the previous experiments, isolation of persisters in the following experiments was performed after 2 h and 6 h of growth, and by addition of 50µg/ml levofloxacin for 24 h. This was done in three independent experiments. Persisters were calculated as the mean percentage that survived levofloxacin treatment of the original CFU/ml in the culture before levofloxacin was added. Results are shown in Fig.17. At most, $4.6x10^6$ CFU/ml of *S.epidermidis* RP62A survived levofloxacin treatment, out of a culture of originally $6.5x10^9$ CFU/ml.

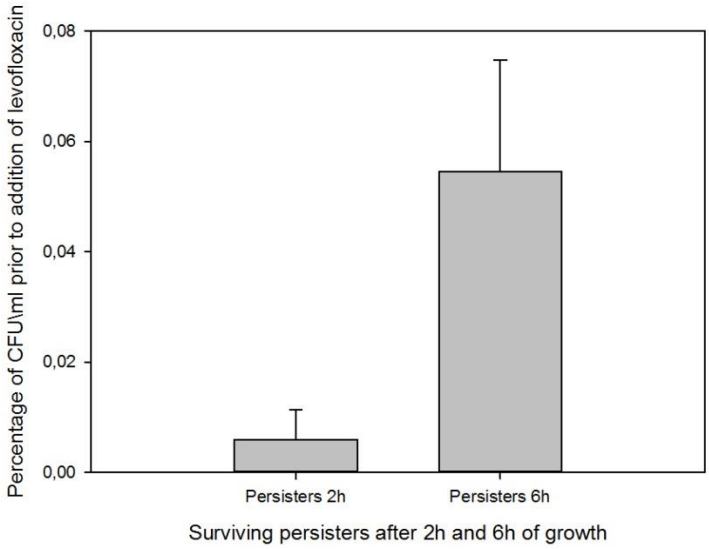

Figure 17: The percentage of *S.epidermidis* RP62A cells that survived 24 h treatment with high concentrations of levofloxacin , added at 2 h and 6 h of growth.

4.4.1 Effect of thiophenone treatment

The effect of thiophenone TF310, TF101 and TF403 on persister development and persister sensitivity to antibiotics were investigated. First, the effect of TF310 on persister development during growth was assayed, by adding TF310 to the culture prior to antibiotic treatment for 24 h. Results from two independent experiments are shown in Fig.18. In Fig.18A and C CFU/ml in the samples pre-treated with TF310 and the samples without any pre-treatment are compared. In all samples except one, 6 h of growth shown in C, the addition of 5µM TF310 did not affect growth of *S.epidermidis* RP62A negatively. In Fig.18B and D, the percentage of the original culture that survived levofloxacin treatment, the persisters, are shown. In the first experiment (Fig.18A and B), there was a higher percentage of persisters in the culture pre-treated with TF310 for 2 h, but not for the culture pre-treated for 6 h. In the second experiment, (Fig.18C and D), the results were opposite. For 2 h the number of persisters was almost the same, with 0 persisters isolated from the untreated sample and 33 CFU/ml persisters in the pre-treated sample. After 6 h the percentage of persisters isolated from the pre-treated culture were higher than for the untreated culture.

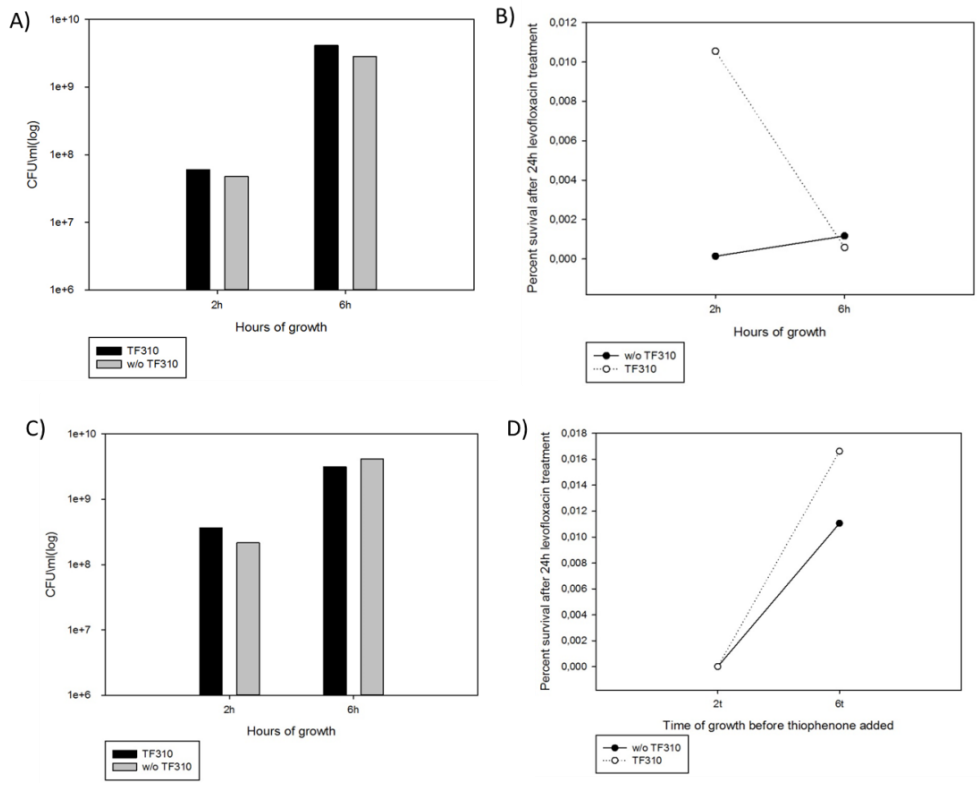

Figure 18: The amount of cells in samples grown with or without 5µM TF310 for 2 or 6 h (A,C). The percentage of cells in each of the samples that survived 24 h treatment with levofloxacin (B,D). A and B together, and C and D together represent one independent experiment

The effect of TF403 was tested in the same way as for TF310, as a pre-treatment added to the culture 2 h or 6 h before levofloxacin was added. Two independent experiments were performed. Result from the first experiment is shown in Fig.19. Original cell number in the samples grown with or without TF403 is shown in Fig.19A. TF403 at 5µM did not affect growth negatively after 2 h of growth, but seemed to have a positive effect on growth. After 6 h CFU/ml had equalized in the two samples, and the sample without TF403 had grown more than the one with TF403 added. CFU/ml surviving levofloxacin treatment is shown in B, and it showed that the amount of cells that survived the levofloxacin treatment was quite high, over $1x10^6$ when isolated after 6 h growth. It also shows that the number of cells that survived the treatment is quite stable, regardless of the amount of cells originally in the culture. The percentage of the different samples that survived the treatment is shown in C). After 2 h there was fewer surviving persisters in the sample pre-treated with TF403, but after 6 h it had changed and the sample pre-treated had higher percentage persisters.

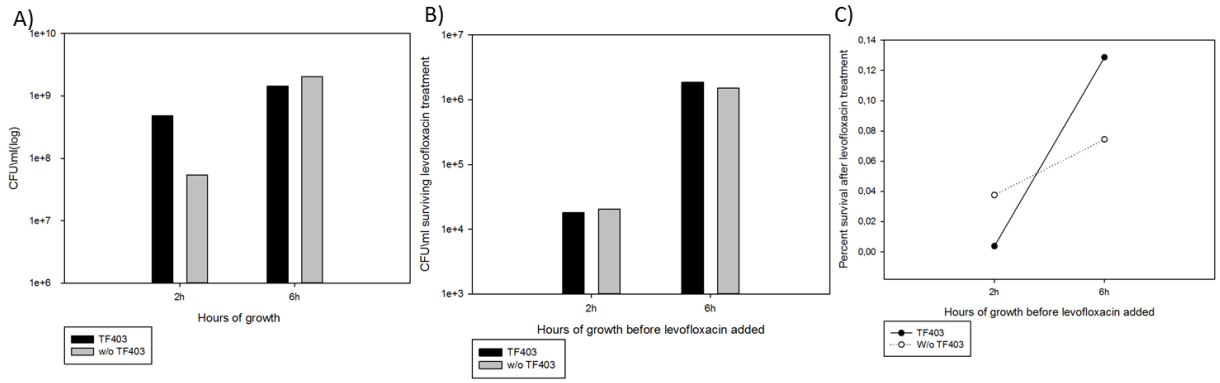

Figure 19: The effect of TF403 as a pre-treament on persister developement/survival compared to an untreated culture. Original CFU/ ml in each culture after 2 h and 6 h of growth A), the amount of isolated persisters B) and the percentage of the original cultures surviving 24 h levofloxacin treatment are shown in C).

Result from a second experiment with TF403 carried out in the same way is shown in Fig.20. Also in this experiment the sample with TF403 added had slightly more growth after 2 h, but after 6 h the untreated sample had the highest CFU/ml (Fig.20A). The number of surviving persisters after 2 h was lower than for the first experiments, but after 6 h the number were quite stable at $1x10^6$ CFU/ml (B). At both time points the untreated sample had more persisters in CFU/ml, despite the fact that the pre-treated sample had a higher CFU/ml after 2 h of growth. This is also reflected when looking at the percentage of original culture surviving levofloxacin treatment (C), as it was more persisters isolated from the untreated sample than

from the TF403 treated sample. After 6 h there were a total of 0,043% persisters isolated from the pre-treated culture and 0,054% from the untreated culture.

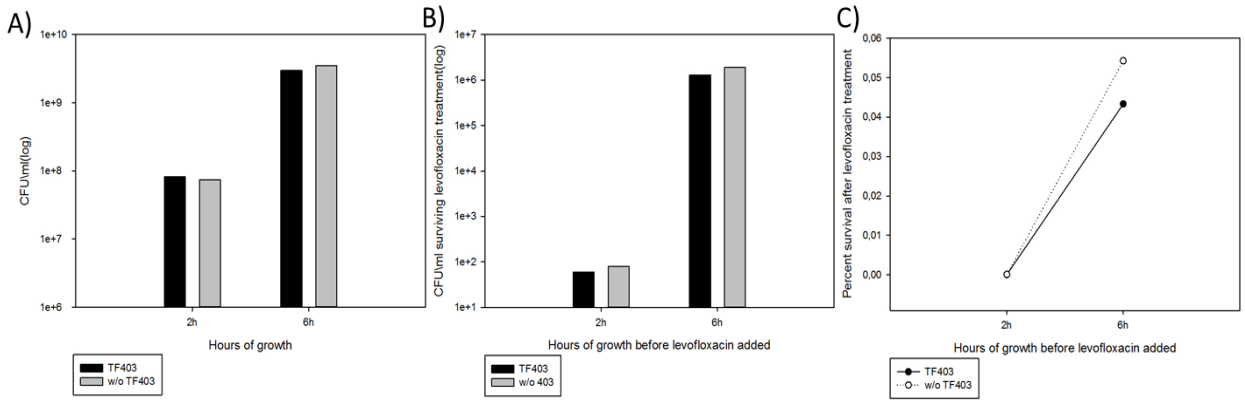

Figure 20: The effect of TF403 as a pre-treament on persister developement/survival compared to an untreated culture. Original CFU/ml in each culture after 2 h and 6 h of growth A), the amount of isolated persisters B) and the percentage of the original cultures surviving 24 h levofloxacin treatment are shown in C).

The effect of TF101 was tested by adding it to the culture at the same time as levofloxacin. This was done in two independent experiments. The results of these two experiments are shown in Fig.21, were graph A and B represents one experiment, and graph C and D the second experiment. In Fig.21A, CFU/ml before removing samples and adding levofloxacin or levofloxacin in combination with TF101 are compared with the number of persisters surviving the different treatments. After both time points the amount of persisters isolated from the culture treated with TF101 in combination with levofloxacin were higher. When looking at the percentage of the original culture surviving the treatment (B), the sample treated with TF101 had the highest percentage of persisters ($3x10^{-4}$ % compared to 0% after 2 h, and 0,1% compared to 0,011% after 6 h).

The second experiment was carried out slightly differently, instead of removing samples from one culture, counting CFU/ml and adding levofloxacin with or without TF101, two different cultures were grown where samples from one got TF101+levofloxacin added, and samples from the other got only levofloxacin added. Accordingly, the original CFU/ml before levofloxacin treatment and TF101 addition is shown in C. There was no major difference in CFU/ml in the two cultures before the treatment. The percentages surviving the different treatments are shown in D. Also in this experiment there were a higher percentage of

surviving persisters in the culture treated with TF101 compared to the untreated culture, but the difference was not as clear as in the first experiment.

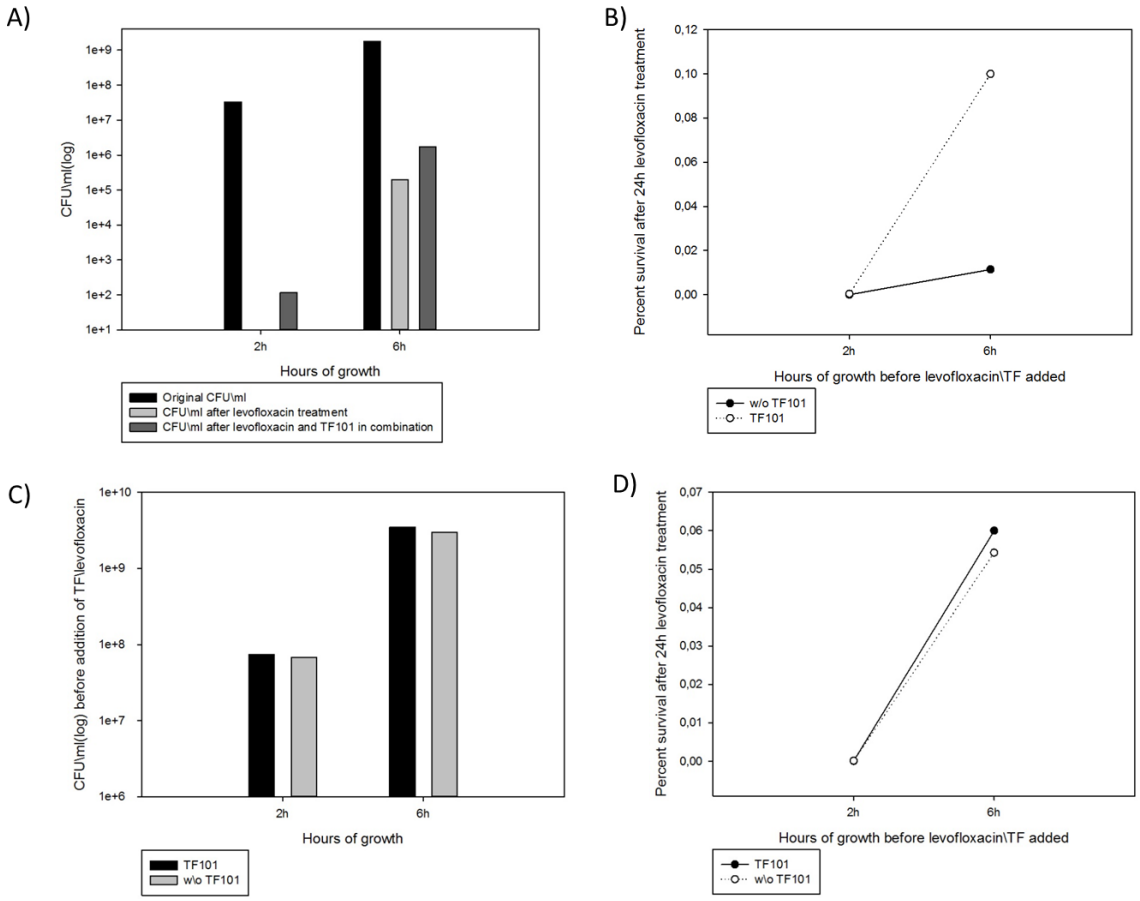

Figure 21: Results from two experiments testing the effect of TF101 on persister survival, A) and B) represent the first experiment, C) and D) the second.

4.5 Adhesion to eukaryotic cells

To look at the effect of three different thiophenones on bacterial adherence to eukaryotic cells, two experiments were carried out with *S.epidermidis* RP62A and Caco-2 cells, with two parallels each time. In the first experiment (Fig.22A) the addition of thiophenones led to more bacteria adhering to the cells. In the second experiment (Fig.22B), there were no clear difference between the cells treated with 5µM thiophenones and the control. Results are presented as CFU per Caco-2 cell.

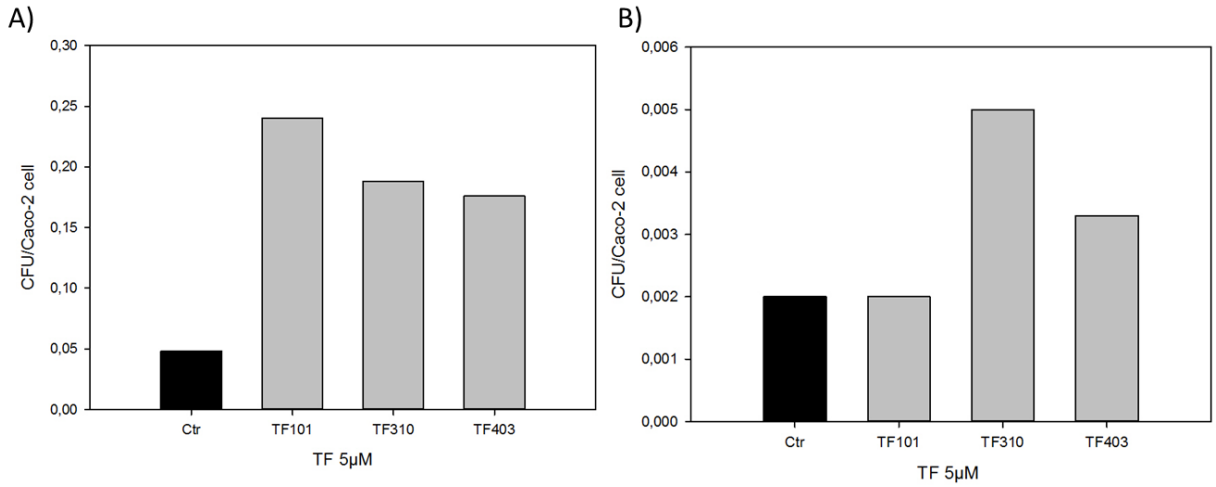

Figure 22: The results of two different experiments investigating the effect of thiophenones on *S.epidermidis* RP62A adherence to eukaryotic Caco-2 cells.

4.6 Infection in C.elegans

To find out whether any of the tested bacterial strains were pathogenic to *C.elegans*, living and dead worms were registered for 7 days after infection with the different bacterial strains. The mean of live worms in each of the 6-wells varied from 13-20 worms, Fig.23.

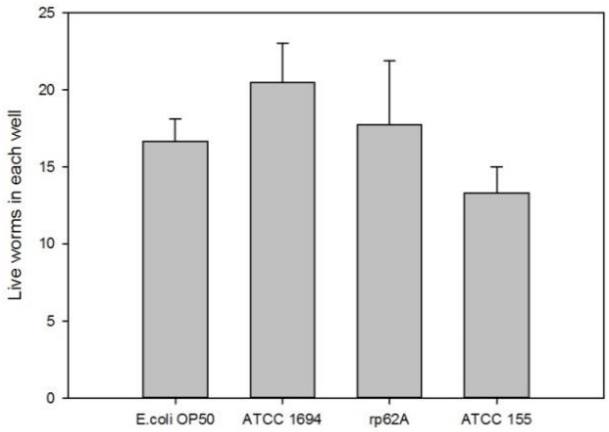

Figure 23: Mean live worms in each well for the different infection plates and control at day of infection.

At day seven the number of dead worms in each infection plate was compared to the amount of live worms at the time of infection (Fig.24). In the control plate containing *E.coli* OP50 the mean number of dead worms was 21.9%. In the infection plate containing *S.aureus* ATCC 1694 the mean number of dead worms was the highest, 38.5%. There were also visual differences between the dead worms which had been feeding on *S.aureus* compared to the other plates. The dead worms were more difficult to spot, because they became almost completely transparent with only the outline showing, and several of them had a rupture in the vulva area. It is worth noticing that the percentage of dead worms was lowest in the plates containing *S.epidermidis* RP62A and ATCC 155, the percentages of dead worms were both lower than the *E.coli*:OP50 control plate. It was decided to carry out an experiment to look at the effect of thiophenones on *S.aureus* infection in *C.elegans*.

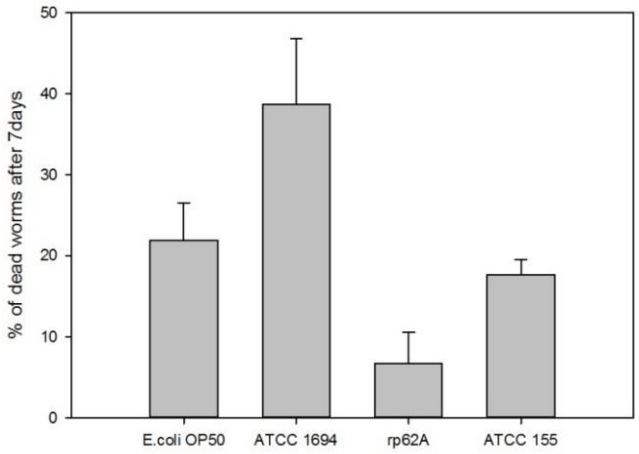

Figure 24: Percentage of dead worms for each of the different infection plates after 7 days of infection.

4.6.1 Effect of thiophenones on infection in C.elegans

Recovery of worms infected with *S.aureus* was followed for 7 days after initial infection. The number of worms was counted at the start of the recovery experiment (Fig.25). The number of worms in each well varied from only 4 to 32 at most. Mean fewest worms was found in the wells were the effect of TF310 on recovery of worms infected by *S.aureus* was being assayed.

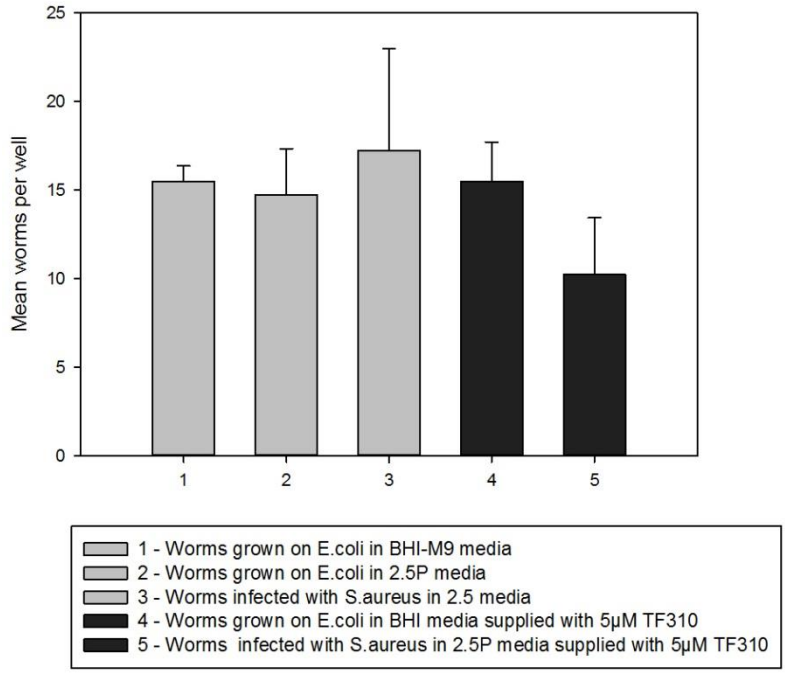

Figure 25: Mean number of worms in the different wells. Black bars indicate wells were the effect of TF310 on recovery was assayed. Error bars represent standard error

Number of live and dead worms in each well was registered every 24 h for 7 days after infection. The percentage of dead worms in each well was calculated. Fig.26 shows the mean percentage of dead worms for each of the samples tested, at day seven after initial infection. Fewest dead worms were found in the wells where *E.coli*:OP50 fed worms were kept in BHI-M9 media. Worms grown in 2.5P media showed higher percentage of death, but this difference was not found to be statistically significant (One way ANOVA). There were not any statistical significant difference between worms infected by *S.aureus* kept in 2.5P media and worms fed with *E.coli* kept in 2.5P media (One way ANOVA). No difference was found between infected worms kept in 2.5P media supplemented with TF310, and infected worms kept in 2.5P without TF310. The highest percentage of dead worms were found among worms kept in BHI-M9 media supplied with TF310, but no statistically significant difference was found when compared to worms kept in BHI-M9 without TF310 (One way ANOVA). Results seem to be due to random sampling variability.

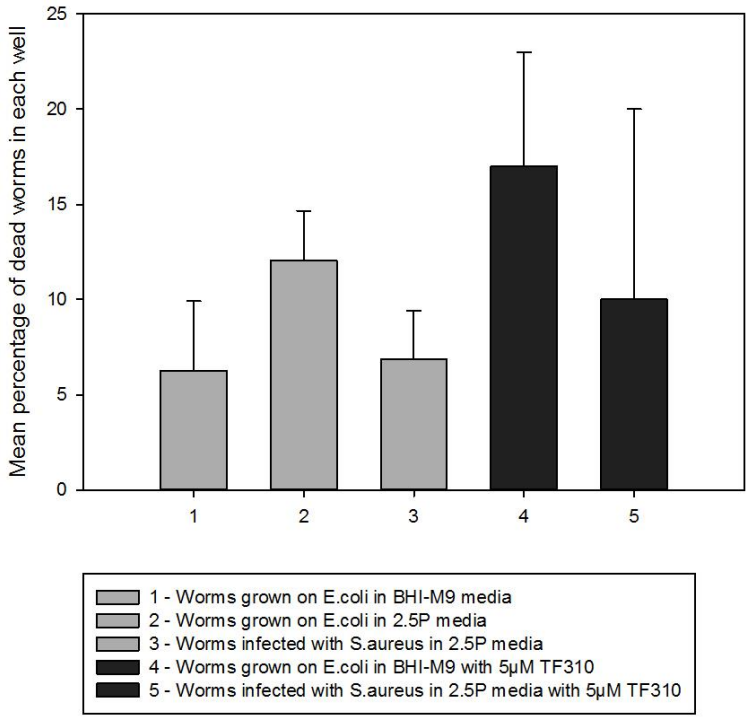

Figure 26: Mean percentage of dead worms in each of the samples. Black bars indicate worms grown in media supplemented with TF310. Error bars represents standard error between the wells in each sample.

5 Discussion

5.1 Thiophenones as quorum sensing inhibitors

The ability of thiophenones 310 and 101 to repress bioluminescence by inhibiting AI-2 mediated communication in *V.harveyi* reporter strains has been shown in several studies (113, 130, 132). In this study the ability of TF301 and TF403 to repress bioluminescence was assayed in *V.harveyi* MM32, an AI-2 reporter strain, together with TF310 and TF101. All thiophenones used in this study repressed bioluminescence induced by DPD by more than 83% at a concentration of only 2.5µM.

5.1.1 AI-2 based quorum sensing in *S.epidermidis*

S.epidermidis RP62A has been sequenced and shown to have the gene for AI-2 synthase, *luxS* (Fig.5). It was therefore interesting to test if a cell-free supernatant prepared from *S.epidermidis* culture could induce bioluminescence in *V.harveyi* MM32 and to look at the effect of thiophenones in inhibiting bioluminescence. The attempt to induce bioluminescence by adding a cell-free *S.epidermidis* supernatant to MM32 was unsuccessful. The experiment was repeated several times with supernatants prepared from different time points, 4 h, 6 h and 16 h of growth, and from different culture media, TSB, HI and BHI. At the same time, bioluminescence induction by DPD was successful. The reason for this could be that the concentration of the AI-2 signaling molecule in the supernatant was too low to induce bioluminescence in the reporter, or that there was no AI-2 production. The time points for preparing the supernatants was influenced by a study on another *S.epidermidis* strain, 1457, that shows that AI-2 production is highest in logarithmic phase, 4-6 h of growth (112). But there could of course be different properties in AI-2 production between these two strains. *S.epidermidis* RP62A cell-free supernatant, prepared after growth overnight in BHI, has in another study been shown to be capable of inducing bioluminescence in a slightly different *V.harveyi* AI-2 reporter strain, BB170 (126). The difference between MM32 and BB170 , is that BB170 has AI-2 production of its own, but when used in bioluminescence assay induction of luminescence in addition to BB170 own baseline production is studied (137, 138). It is therefore a possibility that the concentration of AI-2 in the *S.epidermidis* RP62A supernatant is not high enough to induce bioluminescence when there is no AI-2 production in

the reporter strain, but the concentration is high enough to add to the effect of an AI-2 concentration already near threshold for the induction bioluminescence.

5.2 Thiophenones and biofilm formation

The effect of quorum sensing inhibitors like furanones and thiophenones against biofilm formation has been thoroughly documented in many studies with both gram positive and gram negative bacteria (122, 132, 139, 140). The same has also been shown for both thiophenone and furanone in *S.epidermidis* strain RP62A (ATCC 35984), the same as being used in this study (113, 125, 126). The effect of TF101, TF301, TF310 and TF403 on biofilm formation in *S.epidermidis* RP62A was investigated, with unexpected results. TF403 increased biofilm formation compared to the control, but a small effect could be seen for the other thiophenones. However, none of these results were statistically significant compared to the control. TF101 and TF310 at the same concentration, 5µM, have earlier been reported to inhibit biofilm formation in RP62A with 56% and 59%, respectively (113). Different from this study, culture was prepared in BHI and biofilm was allowed to form with shaking for 6 h, instead of in TSB and without shaking for 6 h. In this thesis the total amount of biofilm formed in the control samples was higher, with an OD_{530nm} reaching 2.2 after 6 h, compared to around 1.5 for the previous study. The different experimental conditions between the two studies could possibly be the reason for the increased biofilm formation and the decreased effect of the thiophenones found here. This is the first experiment showing the effect of TF403 on biofilm formation in *S.epidermidis*. TF403 was as effective as the other thiophenones tested in inhibiting bioluminescence in *V.harveyi* MM32, but had a positive effect on biofilm formation. It is difficult to find an explanation for this, but increased biofilm formation of a *luxS* mutant constructed from *S.epidermidis* strain 1457 have previously been reported and explained by negative regulation of PIA-production by *luxS* (112). It is a possibility that TF403 inhibits AI-2 communication by a different mechanism than the other thiophenones tested, and as a result leads to increased biofilm formation.

5.2.1 Combined effect of thiophenones and antibiotics

The effect of thiophenones in combination with levofloxacin was tested at the same time as the effect of the different thiophenones alone. No additive effect of thiophenones in combination with levofloxacin compared to levofloxacin could be detected, but this is not surprising when we look at the poor effect that thiophenones alone had in inhibiting biofilm formation in this study. It is also worth mentioning the fact that the levofloxacin concentrations tested were all above the MIC-level, and still there were quite a lot of biofilm forming and planktonic growth (results for growth not shown). One of the reasons for this could be that working with such low concentrations and such small volumes of antibiotic solution, increases the risk of inaccuracy, and the possibility that the concentration of antibiotic in each well is below the effective concentration.

5.3 Persister cells

Persister cells were successfully isolated from lag phase to late exponential phase. As antibiotic treatment risks selecting for antibiotic resistant bacteria, the MIC of the isolated cells was tested to confirm that their survival was due to persistence and not resistance. The MIC of isolated persister was higher than for the initial testing, but according to the European Committee on Antimicrobial Susceptibility testing (Eucast) breakpoint tables for interpretation of MICs and zone diameters (2012), MIC-values above 2µg/ml indicate resistance against levofloxacin for Staphylococcus species (141). In accordance with data obtained in another study on *S.epidermidis* RP62A, a persister population was isolated after treatment of high concentrations of levofloxacin (45). At most, a persister population of $4.6x10^6$ CFU/ml was isolated, constituting 0,054% of the original culture.

5.3.1 Quorum sensing inhibition and persister cells

In this thesis an aim was to establish a method for persister isolation and to test the effect of different thiophenones on the formation of persister cells in *S.epidermidis* during growth, and if it had effect on persister cells tolerance to antibiotic. Based on the results, no conclusion could be drawn on the effect of thiophenones, neither during growth nor together with antibiotics. In some of the experiments addition of thiophenones showed increased persister formation/survival, in other experiments the results were opposite. Isolating persisters can be difficult, because they resume regular growth after removal of antibiotic. This was tried to be

circumvented by working quickly and using cold medium for the dilution and plating out process. Nevertheless, this could be a possible factor leading to the non-reproducible results. Another reason could be that the colony counting method to calculate CFU/ml is not sufficiently sensitive and that in many of these experiments very few colonies in each spot were counted, which give increased risk of inaccurate results. The differences observed in some of the experiments between untreated and treated samples could therefore be due to chance. It would be necessary to optimize the method for calculation of persister number, and to repeat the experiments more than two times to be able to make any reliable conclusion.

During the last years one group has in several studies reported that different furanones are able to sensitize *E.coli* and *P.aeruginosa* persister cells to antibiotic (127, 128, 142, 143). In these studies effects of the furanone was assayed using a different method. They isolated the persister cells before adding the selected furanone and incubated for a selected period of time, followed by a second persister isolation. In other words, they tested the effect of furanone on antibiotic sensitivity on already formed persister, while here we investigated the effect of thiophenones on development of persister cells in a growing culture or in a culture challenged with antibiotics. This could be the reason for the differences in the results on the QS-inhibitors effect on persister cells. One of the thiophenones used in this study, TF403, was also the structural homologue of the furanone used in the studies in *E.coli* and *P.aeruginosa* persister cells, BF8, but without proving to have any effect on the amount of surviving persisters in culture. Whether or not persister cell formation is under quorum-sensing control remains unclear. QS-signaling molecules and stationary phase cell-free cultures have shown to increase persister cell number in *P.aeruginosa* (144). In the study on *E.coli* persister cells, the same effect of the furanone was reported in a *luxS* mutant, showing that the furanone might have other targets than AI-2 quorum sensing (127). Optimization of the method is still needed to conclude on the effect of thiophenones on persister cell development and survival.

5.4 Adhesion to eukaryotic cells

An important step in both bacterial pathogenesis and biofilm formation on implant surfaces is the ability to adhere to host cells (29, 145). It was therefore of interest to test the effect of thiophenones in bacterial adherence to eukaryotic cells. No effect of thiophenones could be seen in the two experiments performed. The first experiment could give an indication of thiophenones leading to increased binding, but these results were not reproducible in a second experiment. Over-all, there were few bacteria adhering to each cell, less than a 0.25 bacteria binding to each eukaryotic cell, at most. One of the obvious reasons for this is that *S.epidermidis* is not associated with gut infections, and that another cell line than Caco-2 should be used, which are colon cancer cells. It would therefore be more relevant to test *S.epidermidis* ability to bind to skin or mucosal cells, and the effect of thiophenones in such an experiment. Studies of *S.epidermidis* on adherence to other cell-lines have been conducted. One study have shown that *S.epidermidis* RP62A bind to human umbilical vein endothelial cells (HUVEC), and that about 5.6 % of the inoculum bound to the cells in those experiments (146). In comparison, 0.11 % and 0.0056 % of the inoculum was found to bind to the Caco-2 cells in this study. In the experiment with adherence to HUVEC the highest percentage of adherence was found after 3 h incubation, while we only used 90 min of incubation. Another factor to be considered is the colony counting method. 25µL drops were plated out in one of the experiments, and about 1-3 colonies after a 10^{-1} dilution was obtained, which does not give an accurate calculation of CFU/ml. It would also be of relevance to study the effect that thiophenones alone had on the cells tested, to see if they for instance were toxic to the cells.

5.5 *C.elegans* as a model for infection studies

C.elegans is an economical and easy model used to study microbial infections. The first step was to establish whether any of the selected strains were pathogenic to *C.elegans*. Among the tested strains, *S.aureus* ATCC1694 led to the highest mortality compared to control and other strains tested. This is in line with studies showing that several *S.aureus* strains lead to infection in *C.elegans* (147). Fewer worms died when fed on the *S.epidermidis* strains compared to control. There were also fewer worms, on average in these wells compared to the control. The higher survival of worms in the samples with the lowest number of worms in each well might indicate that worm survival is influenced by stress. This could also bias the

result showing that *S.aureus* led to the highest percentage of death, because the worms feeding on *S.aureus* were also kept in the most crowded environment (Fig.23).

5.5.1 The effect of thiophenones on infection in *C.elegans*

The effect of thiophenones on *C.elegans* recovery from infection by *S.aureus* was investigated in an experiment. No conclusion could be drawn based on the results from this experiment. First of all, *S.aureus* did not seem to have any effect on *C.elegans* survival, compared to worms fed on *E.coli*:OP50. In the previous experiment where the ability to different Staphylococcal strains to cause disease and mortality in *C.elegans* was studied, worms was infected for 7 days, and *S.aureus* was shown to decrease worm survival. In this experiment infection was only carried out for 24 h, before the worms were moved to media supplied with *E.coli*:OP50. It could indicate that 24 h is too little time for *S.aureus* to cause a persistent infection in *C.elegans*. There was also a high variance in the number of worms in each well, and that makes it difficult to draw any conclusion regarding the effect of thiophenones on the worm's recovery from infection. When comparing uninfected worms kept in media with or without thiophenone, no statistically significant difference could be detected. This indicates that 5µM TF310 is not toxic to the worm. In a study where the effect of different compounds against *S.aureus* infection in *C.elegans* was studied, the worms were fed on *S.aureus* in the liquid media during the whole experiment (148). They also used a different strain of S.aureus, causing 100% death of the worms after 4-5 days (148). A more even distribution of the worms in the wells and a longer period of infection prior to the recovery study, or the use of another *S.aureus* strain, will be needed to determine if TF310 has the ability to clear an infection in *C.elegans*.

6 Future studies

- Confirm AI-2 production in *S.epidermidis* by a different method than the bioluminescence assay.

- Test the effect of thiophenones on biofilm formation in combination with the other antibiotics *S.epidermidis* showed susceptibility for; novobiocin, linezolid and vancomycin.

- Find a way to optimize persister quantification, to be able to compare persister formation in cultures grown with or without thiophenones. Use novobiocin, linezolid and vancomycin in isolation experiment, and test the effect of thiophenones in combination with these. Experiment also needs to be repeated several times, to obtain more conclusive results.

- Test the effect of thiophenones on *S.epidermidis* adherence to eukaryotic cells using a cell-line having a more natural implication on *S.epidermidis* infection.

- Optimize infection studies in *C.elegans*, to be able to study the effect of thiophenones on recovery from *S.aureus* infection. This could be done by infecting the worms for a longer period of time than 24 h before recovery studies, or repeat the experiment using a *S.aureus* strain shown to be more virulent against *C.elegans*.

References

1. Raad I, Alrahwan A, Rolston K. Staphylococcus epidermidis: emerging resistance and need for alternative agents. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 1998;26(5):1182-7.
2. Rogers KL, Fey PD, Rupp ME. Coagulase-negative staphylococcal infections. Infectious disease clinics of North America. 2009;23(1):73-98.
3. Otto M. Staphylococcus epidermidis- the "accidental" pathogen. Nature ReviewsMicrobiology. 2009:555-67.
4. Otto M. Staphylococcus epidermidis pathogenesis. Methods in molecular biology (Clifton, NJ). 2014;1106:17-31.
5. Sievert DM, Ricks P, Edwards JR, Schneider A, Patel J, Srinivasan A, et al. Antimicrobial-resistant pathogens associated with healthcare-associated infections: summary of data reported to the National Healthcare Safety Network at the Centers for Disease Control and Prevention, 2009-2010. Infection control and hospital epidemiology : the official journal of the Society of Hospital Epidemiologists of America. 2013;34(1):1-14.
6. Wisplinghoff H, Bischoff T, Tallent SM, Seifert H, Wenzel RP, Edmond MB. Nosocomial bloodstream infections in US hospitals: analysis of 24,179 cases from a prospective nationwide surveillance study. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2004;39(3):309-17.
7. Parvizi J, Pawasarat IM, Azzam KA, Joshi A, Hansen EN, Bozic KJ. Periprosthetic joint infection: the economic impact of methicillin-resistant infections. The Journal of arthroplasty. 2010;25(6 Suppl):103-7.
8. Simon D, Fischer S, Grossman A, Downer C, Hota B, Heroux A, et al. Left ventricular assist device-related infection: treatment and outcome. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2005;40(8):1108-15.
9. Otto M. Staphylococcal Infections: Mechanisms of Biofilm Maturation and Detachment as Critical Determinants of Pathogenicity. Annual review of medicine. 2012.
10. Otto M. Molecular basis of Staphylococcus epidermidis infections. Semin Immunopathol. 2012;34(2):201-14.
11. Gristina AG, Hobgood CD, Webb LX, Myrvik QN. Adhesive colonization of biomaterials and antibiotic resistance. Biomaterials. 1987;8(6):423-6.
12. Ceri H, Olson ME, Stremick C, Read RR, Morck D, Buret A. The Calgary Biofilm Device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms. Journal of clinical microbiology. 1999;37(6):1771-6.
13. Scherr TD, Heim CE, Morrison JM, Kielian T. Hiding in Plain Sight: Interplay between Staphylococcal Biofilms and Host Immunity. Frontiers in immunology. 2014;5:37.
14. Diekema DJ, Pfaller MA, Schmitz FJ, Smayevsky J, Bell J, Jones RN, et al. Survey of infections due to Staphylococcus species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997-1999. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2001;32 Suppl 2:S114-32.
15. Chambers HF. Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications. Clinical microbiology reviews. 1997;10(4):781-91.
16. Mendes RE, Deshpande LM, Costello AJ, Farrell DJ. Molecular epidemiology of Staphylococcus epidermidis clinical isolates from U.S. hospitals. Antimicrobial agents and chemotherapy. 2012;56(9):4656-61.

17. Stefani S, Varaldo PE. Epidemiology of methicillin-resistant staphylococci in Europe. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2003;9(12):1179-86.
18. Bernard L, Hoffmeyer P, Assal M, Vaudaux P, Schrenzel J, Lew D. Trends in the treatment of orthopaedic prosthetic infections. The Journal of antimicrobial chemotherapy. 2004;53(2):127-9.
19. Webb SAR, Kahler CM. Bench-to-bedside review: Bacterial virulence and subversion of host defences. Crit Care. 2008;12(6):8.
20. Sutherland IW. The biofilm matrix--an immobilized but dynamic microbial environment. Trends Microbiol. 2001;9(5):222-7.
21. Costerton JW, Stewart PS, Greenberg EP. Bacterial biofilms: a common cause of persistent infections. Science (New York, NY). 1999;284(5418):1318-22.
22. Costerton W, Veeh R, Shirtliff M, Pasmore M, Post C, Ehrlich G. The application of biofilm science to the study and control of chronic bacterial infections. J Clin Invest. 2003;112(10):1466-77.
23. Lewis K. Persister cells, dormancy and infectious disease. Nat Rev Microbiol. 2007;5(1):48-56.
24. Otto M. Staphylococcal Infections: Mechanisms of Biofilm Maturation and Detachment as Critical Determinants of Pathogenicity. Annu Rev Med. 2013;64:175-88.
25. Yao YF, Sturdevant DE, Otto M. Genomewide analysis of gene expression in Staphylococcus epidermidis biofilms: Insights into the pathophysiology of S-epidermidis biofilms and the role of phenol-soluble modulins in formation of biofilms. J Infect Dis. 2005;191(2):289-98.
26. Otto M. Staphylococcal biofilms. Current topics in microbiology and immunology. 2008;322:207-28.
27. Vacheethasanee K, Temenoff JS, Higashi JM, Gary A, Anderson JM, Bayston R, et al. Bacterial surface properties of clinically isolated Staphylococcus epidermidis strains determine adhesion on polyethylene. J Biomed Mater Res. 1998;42(3):425-32.
28. Carpentier B, Cerf O. Biofilms and their consequences, with particular reference to hygiene in the food industry. The Journal of applied bacteriology. 1993;75(6):499-511.
29. Patti JM, Allen BL, McGavin MJ, Hook M. MSCRAMM-mediated adherence of microorganisms to host tissues. Annu Rev Microbiol. 1994;48:585-617.
30. Bowden MG, Heuck AP, Ponnuraj K, Kolosova E, Choe D, Gurusiddappa S, et al. Evidence for the "dock, lock, and latch" ligand binding mechanism of the staphylococcal microbial surface component recognizing adhesive matrix molecules (MSCRAMM) SdrG. The Journal of biological chemistry. 2008;283(1):638-47.
31. Heilmann C, Hussain M, Peters G, Gotz F. Evidence for autolysin-mediated primary attachment of Staphylococcus epidermidis to a polystyrene surface. Mol Microbiol. 1997;24(5):1013-24.
32. Mack D, Fischer W, Krokotsch A, Leopold K, Hartmann R, Egge H, et al. The intercellular adhesin involved in biofilm accumulation of Staphylococcus epidermidis is a linear beta-1,6-linked glucosaminoglycan: Purification and structural analysis. Journal of bacteriology. 1996;178(1):175-83.
33. Heilmann C, Schweitzer O, Gerke C, Vanittanakom N, Mack D, Gotz F. Molecular basis of intercellular adhesion in the biofilm-forming Staphylococcus epidermidis. Mol Microbiol. 1996;20(5):1083-91.
34. Gerke C, Kraft A, Sussmuth R, Schweitzer O, Gotz F. Characterization of the N-acetylglucosaminyltransferase activity involved in the biosynthesis of the Staphylococcus epidermidis polysaccharide intercellular adhesin. The Journal of biological chemistry. 1998;273(29):18586-93.

35. Costa AR, Henriques M, Oliveira R, Azeredo J. The role of polysaccharide intercellular adhesin (PIA) in Staphylococcus epidermidis adhesion to host tissues and subsequent antibiotic tolerance. Eur J Clin Microbiol Infect Dis. 2009;28(6):623-9.
36. Vuong C, Voyich JM, Fischer ER, Braughton KR, Whitney AR, DeLeo FR, et al. Polysaccharide intercellular adhesin (PIA) protects Staphylococcus epidermidis against major components of the human innate immune system. Cell Microbiol. 2004;6(3):269-75.
37. Costerton JW, Lewandowski Z, Caldwell DE, Korber DR, Lappin-Scott HM. Microbial biofilms. Annu Rev Microbiol. 1995;49:711-45.
38. Sanford BA, deFeijter AW, Wade MH, Thomas VL. A dual fluorescence technique for visualization of Staphylococcus epidermidis biofilm using scanning confocal laser microscopy. J Indust Microbiol. 1996;16(1):48-56.
39. Kong KF, Vuong C, Otto M. Staphylococcus quorum sensing in biofilm formation and infection. International Journal of Medical Microbiology. 2006;296(2-3):133-9.
40. Vuong C, Kocianova S, Yao YF, Carmody AB, Otto M. Increased colonization of indwelling medical devices by quorum-sensing mutants of Staphylococcus epidermidis in vivo. J Infect Dis. 2004;190(8):1498-505.
41. Cohen NR, Lobritz MA, Collins JJ. Microbial Persistence and the Road to Drug Resistance. Cell Host Microbe. 2013;13(6):632-42.
42. Bigger JW. Treatment of staphylococcal infections with penicillin - By intermittent sterilisation. Lancet. 1944;2:497-500.
43. Balaban NQ, Merrin J, Chait R, Kowalik L, Leibler S. Bacterial persistence as a phenotypic switch. Science (New York, NY). 2004;305(5690):1622-5.
44. Keren I, Kaldalu N, Spoering A, Wang YP, Lewis K. Persister cells and tolerance to antimicrobials. FEMS Microbiol Lett. 2004;230(1):13-8.
45. Shapiro JA, Nguyen VL, Chamberlain NR. Evidence for persisters in Staphylococcus epidermidis RP62a planktonic cultures and biofilms. J Med Microbiol. 2011;60(Pt 7):950-60.
46. Lewis K. Persister Cells. In: Gottesman S, Harwood CS, editors. Annual Review of Microbiology, Vol 64, 2010. Annual Review of Microbiology. 64. Palo Alto: Annual Reviews; 2010. p. 357-72.
47. Lewis K. Persister cells: molecular mechanisms related to antibiotic tolerance. Handbook of experimental pharmacology. 2012(211):121-33.
48. Hooper DC. Mechanisms of action of antimicrobials: Focus on fluoroquinolones. Clin Infect Dis. 2001;32:S9-S15.
49. Mulcahy LR, Burns JL, Lory S, Lewis K. Emergence of Pseudomonas aeruginosa Strains Producing High Levels of Persister Cells in Patients with Cystic Fibrosis. Journal of bacteriology. 2010;192(23):6191-9.
50. Govan JRW, Deretic V. Microbial pathogenesis in cystic fibrosis: Mucoid Pseudomonas aeruginosa and Burkholderia cepacia. Microbiol Rev. 1996;60(3):539-+.
51. Hofsteenge N, van Nimwegen E, Silander OK. Quantitative analysis of persister fractions suggests different mechanisms of formation among environmental isolates of E. coli. BMC Microbiol. 2013;13:25.
52. Helaine S, Cheverton AM, Watson KG, Faure LM, Matthews SA, Holden DW. Internalization of Salmonella by Macrophages Induces Formation of Nonreplicating Persisters. Science (New York, NY). 2014;343(6167):204-8.
53. Kint CI, Verstraeten N, Fauvart M, Michiels J. New-found fundamentals of bacterial persistence. Trends in Microbiology. 2012;20(12):577-85.
54. Spoering AL, Lewis K. Biofilms and planktonic cells of Pseudomonas aeruginosa have similar resistance to killing by antimicrobials. Journal of bacteriology. 2001;183(23):6746-51.

55. Fleming A. On the antibacterial action of cultures of a penicillium, with special reference to their use in the isolation of B.influenzae. Brit J Exp Pathol. 1929;10(3):226-36.
56. Zaffiri L, Gardner J, Toledo-Pereyra LH. History of Antibiotics. From Salvarsan to Cephalosporins. J Invest Surg. 2012;25(2):67-77.
57. Alanis AJ. Resistance to antibiotics: are we in the post-antibiotic era? Archives of medical research. 2005;36(6):697-705.
58. Lambert PA. Bacterial resistance to antibiotics: Modified target sites. Advanced Drug Delivery Reviews. 2005;57(10):1471-85.
59. Kohanski MA, Dwyer DJ, Collins JJ. How antibiotics kill bacteria: from targets to networks. Nat Rev Microbiol. 2010;8(6):423-35.
60. Poulikakos P, Falagas ME. Aminoglycoside therapy in infectious diseases. Expert Opin Pharmacother. 2013;14(12):1585-97.
61. Butler MS, Blaskovich MA, Cooper MA. Antibiotics in the clinical pipeline in 2013. Journal of Antibiotics. 2013;66(10):571-91.
62. Bycroft BW, Shute RE. The Molecular-Basis For The Mode Of Action Of Beta-Lactam Antibiotics And Mechanisms Of Resistance. Pharm Res. 1985(1):3-14.
63. Kotra LP, Mobashery S. beta-Lactam antibiotics, beta-lactamases and bacterial resistance. Bull Inst Pasteur. 1998;96(3):139-50.
64. Bolon MK. The Newer Fluoroquinolones. Med Clin N Am. 2011;95(4):793-+.
65. Wimer SM, Schoonover L, Garrison MW. Levofloxacin: A therapeutic review. Clin Ther. 1998;20(6):1049-70.
66. Blondeau JM. Fluoroquinolones: Mechanism of action, classification, and development of resistance. Surv Ophthalmol. 2004;49:S73-S8.
67. Pootoolal J, Neu J, Wright GD. Glycopeptide antibiotic resistance. Annu Rev Pharmacol Toxicol. 2002;42:381-408.
68. Lundstrom TS, Sobel JD. Antibiotics for gram-positive bacterial infections: vancomycin, quinupristin-dalfopristin, linezolid, and daptomycin. Infectious disease clinics of North America. 2004;18(3):651-+.
69. Levine DP. Vancomycin: A history. Clin Infect Dis. 2006;42:S5-S12.
70. Karchmer AW, Archer GL, Dismukes WE. Staphylococcus epidermidis causing prosthetic valve endocarditis- Microbiologic and clinical observations as guides to therapy Ann Intern Med. 1983;98(4):447-55.
71. Nailor MD, Sobel JD. Antibiotics for Gram-Positive Bacterial Infections: Vancomycin, Teicoplanin, Quinupristin/Dalfopristin, Oxazolidinones, Daptomycin, Dalbavancin, and Telavancin. Infectious disease clinics of North America. 2009;23(4):965-+.
72. Heide L. The aminocoumarins: biosynthesis and biology. Nat Prod Rep. 2009;26(10):1241-50.
73. Gormley NA, Orphanides G, Meyer A, Cullis PM, Maxwell A. The interaction of coumarin antibiotics with fragments of the DNA gyrase B protein. Biochemistry. 1996;35(15):5083-92.
74. Anderle C, Stieger M, Burrell M, Reinelt S, Maxwell A, Page M, et al. Biological activities of novel gyrase inhibitors of the aminocoumarin class. Antimicrobial agents and chemotherapy. 2008;52(6):1982-90.
75. Devasahayam G, Scheld WM, Hoffman PS. Newer antibacterial drugs for a new century. Expert Opin Investig Drugs. 2010;19(2):215-34.
76. Brandi L, Fabbretti A, Pon CL, Dahlberg AE, Gualerzi CO. Initiation of protein synthesis: a target for antimicrobials. Expert Opin Ther Targets. 2008;12(5):519-34.
77. Fung HB, Kirschenbaum HL, Ojofeitimi BO. Linezolid: An oxazolidinone antimicrobial agent. Clin Ther. 2001;23(3):356-91.

78. Wilson DN, Nierhaus KH. The oxazolidinone class of drugs find their orientation on the ribosome. Mol Cell. 2007;26(4):460-2.
79. Jones RN, Ballow CH, Biedenbach DJ, Ctr ZSGM. Multi-laboratory assessment of the linezolid spectrum of activity using the Kirby-Bauer disk diffusion method: Report of the Zyvox (R) Antimicrobial Potency Study (ZAPS) in the United States. Diagnostic microbiology and infectious disease. 2001;40(1-2):59-66.
80. Orsi GB, Falcone M, Venditti M. Surveillance and management of multidrug-resistant microorganisms. Expert Review of Anti-Infective Therapy. 2011;9(8):653-79.
81. Kumar A, Schweizer HP. Bacterial resistance to antibiotics: Active efflux and reduced uptake. Advanced Drug Delivery Reviews. 2005;57(10):1486-513.
82. Madigan M MJ, Stahl D , Clark D. Brock Biology of Microorganisms. 13th ed: Pearson; 2011.
83. Hawkey PM, Jones AM. The changing epidemiology of resistance. The Journal of antimicrobial chemotherapy. 2009;64 Suppl 1:i3-10.
84. Kirby WM. Extraction Of A Highly Potent Penicillin Inactivator From Penicillin Resistant Staphylococci. Science (New York, NY). 1944;99(2579):452-3.
85. Smyth DS, Wong A, Robinson DA. Cross-species spread of SCCmec IV subtypes in staphylococci. Infect Genet Evol. 2011;11(2):446-53.
86. Grundmann H, Aires-De-Sousa M, Boyce J, Tiemersma E. Emergence and resurgence of meticillin-resistant Staphylococcus aureus as a public-health threat. Lancet. 2006;368(9538):874-85.
87. Cetinkaya Y, Falk P, Mayhall CG. Vancomycin-resistant enterococci. Clinical microbiology reviews. 2000;13(4):686-+.
88. Paulsen IT, Banerjei L, Myers GSA, Nelson KE, Seshadri R, Read TD, et al. Role of mobile DNA in the evolution of vancomycin-resistant Enterococcus faecalis. Science (New York, NY). 2003;299(5615):2071-4.
89. Uttley AHC, Collins CH, Naidoo J, George RC. Vancomycin-Resistant Enterococci. Lancet. 1988;1(8575-6):57-8.
90. Waters CM, Bassler BL. Quorum sensing: cell-to-cell communication in bacteria. Annual review of cell and developmental biology. 2005;21:319-46.
91. Zhu J, Miller MB, Vance RE, Dziejman M, Bassler BL, Mekalanos JJ. Quorum-sensing regulators control virulence gene expression in Vibrio cholerae. Proc Natl Acad Sci U S A. 2002;99(5):3129-34.
92. Vuong C, Gerke C, Somerville GA, Fischer ER, Otto M. Quorum-sensing control of biofilm factors in Staphylococcus epidermidis. J Infect Dis. 2003;188(5):706-18.
93. Ng WL, Bassler BL. Bacterial Quorum-Sensing Network Architectures. Annual Review of Genetics. Annual Review of Genetics. 43. Palo Alto: Annual Reviews; 2009. p. 197-222.
94. Li YH, Tian X. Quorum sensing and bacterial social interactions in biofilms. Sensors (Basel, Switzerland). 2012;12(3):2519-38.
95. Nealson KH, Hastings JW. Bacterial bioluminescence - Its control and ecological significance. Microbiol Rev. 1979;43(4):496-518.
96. Federle MJ, Bassler BL. Interspecies communication in bacteria. J Clin Invest. 2003;112(9):1291-9.
97. Gray KM, Passador L, Iglewski BH, Greenberg EP. Interchangeability and specificity of components from the quorum-sensing regulatory systems of Vibrio fischeri and Pseudomonas aeruginosa. Journal of bacteriology. 1994;176(10):3076-80.
98. Fuqua C, Greenberg EP. Listening in on bacteria: Acyl-homoserine lactone signalling. Nat Rev Mol Cell Biol. 2002;3(9):685-95.

99. Latifi A, Winson MK, Foglino M, Bycroft BW, Stewart GS, Lazdunski A, et al. Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in Pseudomonas aeruginosa PAO1. Mol Microbiol. 1995;17(2):333-43.
100. Davies DG, Parsek MR, Pearson JP, Iglewski BH, Costerton JW, Greenberg EP. The involvement of cell-to-cell signals in the development of a bacterial biofilm. Science (New York, NY). 1998;280(5361):295-8.
101. Sturme MHJ, Kleerebezem M, Nakayama J, Akkermans ADL, Vaughan EE, de Vos WM. Cell to cell communication by autoinducing peptides in gram-positive bacteria. Antonie Van Leeuwenhoek. 2002;81(1-4):233-43.
102. Kleerebezem M, Quadri LEN, Kuipers OP, deVos WM. Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. Mol Microbiol. 1997;24(5):895-904.
103. Novick RP, Geisinger E. Quorum Sensing in Staphylococci. Annual Review of Genetics. Annual Review of Genetics. 42. Palo Alto: Annual Reviews; 2008. p. 541-64.
104. Otto M, Sussmuth R, Jung G, Gotz F. Structure of the pheromone peptide of the Staphylococcus epidermidis agr system. Febs Letters. 1998;424(1-2):89-94.
105. Pereira CS, Thompson JA, Xavier KB. AI-2-mediated signalling in bacteria. Fems Microbiology Reviews. 2013;37(2):156-81.
106. Schauder S, Shokat K, Surette MG, Bassler BL. The LuxS family of bacterial autoinducers: biosynthesis of a novel quorum-sensing signal molecule. Mol Microbiol. 2001;41(2):463-76.
107. Miller ST, Xavier KB, Campagna SR, Taga ME, Semmelhack MF, Bassler BL, et al. Salmonella typhimurium recognizes a chemically distinct form of the bacterial quorum-sensing signal Al-2. Mol Cell. 2004;15(5):677-87.
108. Sun J, Daniel R, Wagner-Dobler I, Zeng AP. Is autoinducer-2 a universal signal for interspecies communication: a comparative genomic and phylogenetic analysis of the synthesis and signal transduction pathways. BMC Evol Biol. 2004;4:36.
109. Bassler BL, Wright M, Showalter RE, Silverman MR. Intercellular signalling in Vibrio-harveyi - Sequence and function of genes regulating expression of luminescence. Mol Microbiol. 1993;9(4):773-86.
110. Bassler BL, Greenberg EP, Stevens AM. Cross-species induction of luminescence in the quorum-sensing bacterium Vibrio harveyi. Journal of bacteriology. 1997;179(12):4043-5.
111. Genbank. Staphylococcus epidermidis RP62A, complete genome http://www.ncbi.nlm.nih.gov/nuccore/57636010?from=1781118&to=1781588&sat=4&sat_key=105780764.
112. Xu L, Li HL, Vuong C, Vadyvaloo V, Wang JP, Yao YF, et al. Role of the luxS quorum-sensing system in biofilm formation and virulence of Staphylococcus epidermidis. Infect Immun. 2006;74(1):488-96.
113. Lonn-Stensrud J, Naemi AO, Benneche T, Petersen FC, Scheie AA. Thiophenones inhibit Staphylococcus epidermidis biofilm formation at nontoxic concentrations. FEMS immunology and medical microbiology. 2012;65(2):326-34.
114. Li M, Villaruz AE, Vadyvaloo V, Sturdevant DE, Otto M. Al-2-dependent gene regulation in Staphylococcus epidermidis. BMC Microbiol. 2008;8:9.
115. Mehlin C, Headley CM, Klebanoff S. An inflammatory polypeptide complex from Staphylococcus epidermidis: Isolation and characterization. J Exp Med. 1999;189(6):907-17.
116. Hentzer M, Givskov M. Pharmacological inhibition of quorum sensing for the treatment of chronic bacterial infections. J Clin Invest. 2003;112(9):1300-7.
117. de Nys R, Wright AD, König GM, Sticher O. New halogenated furanones from the marine alga delisea pulchra (cf. fimbriata). Tetrahedron. 1993;49(48):11213-20.

118. Givskov M, de Nys R, Manefield M, Gram L, Maximilien R, Eberl L, et al. Eukaryotic interference with homoserine lactone-mediated prokaryotic signalling. Journal of bacteriology. 1996;178(22):6618-22.
119. Manefield M, de Nys R, Kumar N, Read R, Givskov M, Steinberg P, et al. Evidence that halogenated furanones from Delisea pulchra inhibit acylated homoserine lactone (AHL)-mediated gene expression by displacing the AHL signal from its receptor protein. Microbiology-Uk. 1999;145:283-91.
120. Givskov M, DeNys R, Manefield M, Gram L, Maximilien R, Eberl L, et al. Eukaryotic interference with homoserine lactone-mediated prokaryotic signaling. Journal of bacteriology. 1996;178(22):6618-22.
121. Manefield M, Harris L, Rice SA, De Nys R, Kjelleberg S. Inhibition of luminescence and virulence in the black tiger prawn (Penaeus monodon) pathogen Vibrio harveyi by intercellular signal antagonists. Applied and environmental microbiology. 2000;66(5):2079-84.
122. Ren DC, Sims JJ, Wood TK. Inhibition of biofilm formation and swarming of Escherichia coli by (5Z)-4-bromo-5(bromomethylene)-3-butyl-2(5H)-furanone. Environ Microbiol. 2001;3(11):731-6.
123. de Nys R, Givskov M, Kumar N, Kjelleberg S, Steinberg PD. Furanones. Progress in molecular and subcellular biology. 2006;42:55-86.
124. Hentzer M, Wu H, Andersen JB, Riedel K, Rasmussen TB, Bagge N, et al. Attenuation of Pseudomonas aeruginosa virulence by quorum sensing inhibitors. Embo Journal. 2003;22(15):3803-15.
125. Baveja JK, Willcox MD, Hume EB, Kumar N, Odell R, Poole-Warren LA. Furanones as potential anti-bacterial coatings on biomaterials. Biomaterials. 2004;25(20):5003-12.
126. Lonn-Stensrud J, Landin MA, Benneche T, Petersen FC, Scheie AA. Furanones, potential agents for preventing Staphylococcus epidermidis biofilm infections? J Antimicrob Chemother. 2009;63(2):309-16.
127. Pan JC, Xie X, Tian W, Bahar AA, Lin N, Song FC, et al. (Z)-4-Bromo-5-(bromomethylene)-3-methylfuran-2(5H)-one sensitizes Escherichia coli persister cells to antibiotics. Appl Microbiol Biotechnol. 2013;97(20):9145-54.
128. Pan JC, Bahar AA, Syed H, Ren DC. Reverting Antibiotic Tolerance of Pseudomonas aeruginosa PAO1 Persister Cells by (Z)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5H)-one. Plos One. 2012;7(9):9.
129. Benneche T, Herstad G, Rosenberg M, Assev S, Scheie AA. Facile synthesis of 5-(alkylidene)thiophen-2(5H)-ones. A new class of antimicrobial agents. RSC Adv. 2011;1(2):323-32.
130. Defoirdt T, Benneche T, Brackman G, Coenye T, Sorgeloos P, Scheie AA. A Quorum Sensing-Disrupting Brominated Thiophenone with a Promising Therapeutic Potential to Treat Luminescent Vibriosis. Plos One. 2012;7(7):7.
131. Defoirdt T, Crab R, Wood TK, Sorgeloos P, Verstraete W, Bossier P. Quorum sensing-disrupting brominated furanones protect the gnotobiotic brine shrimp Artemia franciscana from pathogenic Vibrio harveyi, Vibrio campbellii, and Vibrio parahaemolyticus isolates. Applied and environmental microbiology. 2006;72(9):6419-23.
132. Witso IL, Benneche T, Vestby LK, Nesse LL, Lonn-Stensrud J, Scheie AA. Thiophenone and furanone in control of Escherichia coli O103:H2 virulence. Pathogens and disease. 2014.
133. Benneche T, Chamgordani EJ, Scheie AA. Reaction of (Z)-5-(Bromomethylene)thiophen-2(5 H)-one with some nucleophiles in search for new biofilm inhibitors. Synthetic Communications. 2013;43(3):431-7.

134. Wu X, Santos RR, Fink-Gremmels J. Staphylococcus epidermidis biofilm quantification: Effect of different solvents and dyes. Journal of microbiological methods. 2014;101:63-6.
135. Chen CY, Nace GW, Irwin PL. A 6 x 6 drop plate method for simultaneous colony counting and MPN enumeration of Campylobacter jejuni, Listeria monocytogenes, and Escherichia coli. Journal of microbiological methods. 2003;55(2):475-9.
136. Wiegand I, Hilpert K, Hancock RE. Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. Nature protocols. 2008;3(2):163-75.
137. Surette MG, Bassler BL. Quorum sensing in Escherichia coli and Salmonella typhimurium. Proc Natl Acad Sci U S A. 1998;95(12):7046-50.
138. DeKeersmaecker SC, Vanderleyden J. Constraints on detection of autoinducer-2 (AI-2) signalling molecules using Vibrio harveyi as a reporter. Microbiology (Reading, England). 2003;149(Pt 8):1953-6.
139. Lonn-Stensrud J, Petersen FC, Benneche T, Aamdal SA. Synthetic bromated furanone inhibits autoinducer-2-mediated communication and biofilm formation in oral streptococci. Oral Microbiol Immunol. 2007;22(5):340-6.
140. Vestby LK, Johannesen KCS, Witso IL, Habimana O, Scheie AA, Urdahl AM, et al. Synthetic brominated furanone F202 prevents biofilm formation by potentially human pathogenic Escherichia coli O103: H2 and Salmonella ser. Agona on abiotic surfaces. Journal of applied microbiology. 2014;116(2):258-68.
141. Testing ECoAS. Breakpoint tables for interpretation of MICs and zone diameters. http://www.webcitation.org/query.php?url=http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Disk_test_documents/EUCAST_breakpoints_v_2.0_120101.pdf&refdoi=10.1186/1476-0711-12-412012.
142. Pan J, Ren D. Structural effects on persister control by brominated furanones. Bioorganic & medicinal chemistry letters. 2013;23(24):6559-62.
143. Pan J, Song F, Ren D. Controlling persister cells of Pseudomonas aeruginosa PDO300 by (Z)-4-bromo-5-(bromomethylene)-3-methylfuran-2(5H)-one. Bioorganic & medicinal chemistry letters. 2013;23(16):4648-51.
144. Moker N, Dean CR, Tao J. Pseudomonas aeruginosa increases formation of multidrug-tolerant persister cells in response to quorum-sensing signaling molecules. Journal of bacteriology. 2010;192(7):1946-55.
145. Heilmann C. Adhesion mechanisms of staphylococci. Advances in experimental medicine and biology. 2011;715:105-23.
146. Merkel GJ, Scofield BA. Interaction of Staphylococcus epidermidis with endothelial cells in vitro. Med Microbiol Immunol. 2001;189(4):217-23.
147. Sifri CD, Begun J, Ausubel FM, Calderwood SB. Caenorhabditis elegans as a model host for Staphylococcus aureus pathogenesis. Infect Immun. 2003;71(4):2208-17.
148. Kong C, Yehye WA, Abd Rahman N, Tan MW, Nathan S. Discovery of potential anti-infectives against Staphylococcus aureus using a Caenorhabditis elegans infection model. BMC complementary and alternative medicine. 2014;14:4.

Supplementary

M9-buffer

1.5g	1M KH^2 pH 4
3g	1M Na_2H(1?)
2.5g	NaCl
500ml	d^2H_2O

The buffer was autoclaved for 15 min at 121°C, and 500µl 1M $MgSO^4$ pH4 was added right before use.

Nematode growth medium (NGM)-plates

8.5g	Agar
1.25g	Peptone
1.5g	NaCl
487ml	d^2H_2O

The NGM-solution was autoclaved for 15 min at 121°C and cooled down to 56°C in a water bath. Right before it was poured on to the plates, the following was added:

12.5ml	1M $CaCl_2$
500µl	Cholesterol, 5mg\ml
500µl	1M KH_2 pH4
500µl	1M $MgSO^4$

BHI20- Infection plates

4.5g	Agar
1.85g	BHI
1.5g	Peptone
0.1g	NaCl
244ml	d^2H_2O

The solution was autoclaved for 15 min at 121°C, and cooled down to 56°C in a water bath. Before pouring 5ml of the solution into each well of a 6-well plate, the following was added:

5ml	1M $CaCl_2$
200µl	Cholesterol, 5mg\ml
200µl	1M KH_2 pH4
200µl	1M $MgSO^4$

Optimized Buffered Peptone Water (2.5P-media)

1.25g	Peptone
1.50g	NaCl
1.75g	Disodium phosphate
0.75g	Postassium dihydrogen phosphate
500ml	d^2H_2O

The solution was autoclaved for 15 min at 121°C, and cooled down before the following was added:

500 µl	$CaCl_2$(1 M)
500 µl	Cholesterol (5mg/ml)
500 µl	$MgSO_4$ (1 M)

BA-medium for bioluminescence assay

4.39g/0.3M	NaCl
3.01g/0.05M	$MgSO^4$
0.5g/0.2%	Casamino acid
250ml	d^2H_2O

pH was calibrated to pH 7.5 with 1M KOH, and it was autoclaved for 15 min at 121°C.

After the medium was cooled down the following was added:

0.34g in 2.5ml d^2H_2O	1M Potassiumphosphate diHydrogen
0.0435g in 2.5ml d^2H_2O	0.1M L-arginine
2.5ml	Glycerol

The medium was prepared right before an experiment and the rest was kept in a -80°C freezer to keep it fresh.

Before inoculation of bacteria 0.1g riboflavin in 10ml d^2H_2O and 0.05g thiamine in 10ml d^2H_2O was sterile filtrated with a 0,2µm celluloseacetate filter (Whatman®) and diluted 500 and 10 times, respectively, and 1µl\ml of each was added to the medium.